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PRINCIPAL INVESTIGATOR: Dezhong J. Liao

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057

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Dezhong J. Liao

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University Medical Center
Washington, DC 20057

E-Mail:

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This project aims to elucidate the cell cycle basis on which c-*myc* oncogene induces mammary carcinogenesis and transforming growth factor α (*tgfα*) promotes the process. Conduction of this training project will help the P.I. to develop his career in breast cancer research. Data obtained so far have shown that c-*myc* transgenic mammary tumors may develop specific foci that are more aggressive than their adjacent tumor areas and thus represent a second stage of tumor progression. c-Myc may induce E2F1 and cyclin A2 to initiate the tumor development, whereas overexpression of cyclins D1 and E may occur as later events to promote tumor progression to a more aggressive phenotype. TGFα may enhance c-Myc-induced mammary carcinogenesis by inducing cyclin D1 and facilitating the loss of pRB expression, resulting in an earlier development of more aggressive tumors in *tgfα*/c-*myc* double transgeneic mice, compared to the c-*myc* tumors. These findings in the transgenic animals may have relevance to human breast cancer.

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INTRODUCTION:

This subject of study aims to elucidate the cell cycle basis on which c-*myc* oncogene initiates mammary carcinogenesis and transforming growth factor α (*tgf*α) promotes the process. Conduction of this training project will help the P.I., Dr. Dezhong Liao, to develop his career in breast cancer research. This research project has four tasks, each of which is consisted of two parts, i.e. *in vivo* studies with transgenic mice and *in vitro* studies with cultured cells. The four tasks are:

- Task 1. Study whether expression of E2F1 and cyclin A2 is induced to mediate mammary cancer formation in c-*myc* mice. (Months 1-15).
- Task 2. Investigate whether c-*myc* initially suppresses cyclin D1 expression, but overexpression of cyclins D1 and E occurs as later events to facilitate progression of tumors to faster growing phenotypes. (Months 1-15)
- Task 3. Study the mechanisms and the roles of overexpression of cyclins D1 and E in mammary carcinogenesis in TGFα/c-*myc* mice. (Months 16-30).
- Task 4. Investigate the mechanisms for the loss of pRB during mammary carcinogenesis in *myc* and TGFα/c-*myc* mice. (Months 31-36)

BODY:

1. *In vivo* animal experiments on the cell cycle basis of c-*myc*-induced, *tgf*α-enhanced mouse mammary carcinogenesis: We have actually started the project since this proposal was submitted to DOD in June of 1999, about one year earlier than the time when the award was started. During the past two years, most *in vivo* animal experiments have been completed. The main results were published in one research data report (1) and one review article (2). DOD was not acknowledged in these two publications, because they were submitted for publication before this award was started. Nevertheless, the results published in these two papers (1,2) accomplished tasks 1a, 1d, 2d, 3a, 3e, 4b and part of 4a proposed in the Statement of Work (SOW), although some of these tasks were originally scheduled for the second and third years of this award. The results are summarized below:

Abstract (summaries of appendices I and II):
In c-*myc* transgenic mice, c-*myc* expression was high in the hyperplastic mammary epithelium and the majority of tumor areas. However, the c-*myc* tumors displayed focal areas that showed a weaker staining of hematoxylin, a lower expression of c-*myc*, a lower percentage of dead cells, but a higher rate of cellular proliferation, compared to the adjacent tumor areas. These "tumor-within-a-tumor foci" were observed only in some large, advanced tumors, but not in early tumors; thus, their appearance may represent a second step of tumor progression. Therefore, the c-*myc* mouse may be the first animal model of mammary carcinogenesis that shows recognizable multistages of tumor progression. Expression of E2F1 and cyclin A2 was highly induced in the hyperplastic mammary glands and tumors, and was co-localized with c-*myc* expression. In contrast, expression of cyclins D1 and E occurred only in the tumor foci, indicating a reciprocal relationship in the expression between c-*myc* and cyclins D1 and E. Overexpression of cyclin D1 also occurred in the hyperplastic mammary glands in *tgf*α-trasngenic mice, and

in the mammary tumors from *tgfα*/c-*myc* double transgenic mice, without showing reciprocal relationship to the c-*myc* expression. In contrast to c-*myc* tumors, most *tgfα*/c-*myc* tumors showed undetectable levels of retinoblastoma protein (pRB), and the loss of pRB occurred in some cases at the mRNA level. These results suggest that E2F1 and cyclin A2 may be induced by c-Myc to mediate the onset of mammary cancer, whereas overexpression of cyclins D1 and E may occur later to facilitate tumor progression. TGFα may play its synergistic role, at least in part, by inducing cyclin D1 and facilitating the loss of pRB.

2. Cell death properties of c-Myc-induced mouse mammary tumors: During our studies on the cell cycle profile of the c-*myc* tumors, we found that about 15% of the cells were dead via programmed cell death, in strong contrast to only 1-2% in *tgfα*/c-*myc* double transgenic tumors (1,2). We therefore further investigate the cell death properties in the c-*myc* tumor tissue (3), although this work was not originally planed in the proposal. The results were summarized below:

Abstract (summary of appendix III):
Enforced expression of c-*myc* has been shown to serve as an apoptotic stimulus in cultured cells, in the absence of survival factors. Prior studies have also demonstrated that tissues expressing the c-*myc* transgene display a large number of dead cells. In this study, we found that MMTV-c-*myc* transgenic mouse mammary tumor cells exhibited mitochondrial malformation, characterized by a primarily amorphous matrix, with very few cristae. Mitochondria were also frequently degenerated. Cytochrome c expression was much lower in the majority of MMTV-c-*myc* mammary tumor cells, compared to adjacent, c-*myc*-silenced tumor foci. In the majority of the tumor areas, there were many dying and dead cells organized in clusters, termed dead cell islands. These cells exhibited shrinkage, TUNEL positive staining, nuclear localization of apoptosis-inducing factor (AIF), but a lack of typical apoptotic morphology, such as nuclear condensation and formation of cell membrane blebs and apoptotic bodies. Many macrophages were detected infiltrating into these dead cell islands and engulfing the dying or dead tumor cells. Disruption of tumor cells was rare, but disrupted macrophages were common. These morphologic features suggest that the atypical cell death in c-*myc* transgenic mammary tumor tissue may be related to malformed and degenerated mitochondria, possibly leading to energy deficiency, and to the early involvement of macrophages engulfing the dying cells.

3. In vitro experiments with cell culture: Tasks 1b, 1c, 2a, 2b and 2c of SOW were originally scheduled for this time period. We have initiated these experiments by requesting the relevant cDNAs (cDNAs of c-myc, e2f1. and cyclins A2, D1 and E) from different laboratories that cloned these genes. Some of the cDNAs have also been transfected into bacteria for propagation. However, we later learned that the CommaD1 and HC14MV4 cells contain mutations of the p53 tumor suppressor gene, which affect significantly the cell cycle regulation. Hence, these cells may have quite different cell cycle properties, compared to the normal mammary epithelium. Because of this unexpected development, we did not continue to use Commad1 and HC14MV4 cells in our *in vitro* studies, which affects the progress of tasks 1b, 1c, 2a, 2b and 2c. To

accomplish these tasks, we need to establish or obtain new mouse mammary epithelial cell lines. While we are working on isolation of mammary epithelial cells from c-*myc* transgenic mice, we postpone these tasks to the second period of this award, and conduct in advance tasks 1d, 2d, 3a, 3e, 4a and 4b that were originally scheduled for years two and three.

4. Study of c-*myc* amplification and overexpession in human breast cancer biopsies: It is important to investigate the relevance of our findings in c-*myc* transgenic model to the human breast cancer, although studies on human subjects were not planed in the proposal. Therefore we studied amplification and expression of c-*myc* gene in a group of high-grade human breast cancer biopsies (4). The results were summarized below:

Abstract (summary of appendix IV):
Although there have been many reports on the amplification and overexpression of the c-*myc* oncogene in human breast cancer, few of such studies have utilized *in situ* hybridization approaches to directly analyze the gene amplification and RNA expression on tumor tissue sections. In this study, we analyzed gene amplification, RNA expression, and protein expression of the c-*myc* gene in tissue specimens of high-grade breast cancer with these techniques. A surprisingly high proportion (70%) of the tumor cases was found to demonstrate amplification of the c-*myc* gene. However, the level of amplification was surprisingly low, ranging between 1-4 copies of gene gains, and the majority (84%) of the cases with the gene amplification gained only 1-2 copies. Approximately 95% and 79% of the cases exhibited c-*myc* RNA and protein overexpression, respectively; in contrast, reduction mammoplasty-derived breast tissue expressed the mRNA and the protein only in about 14% (1 of 7) and 0% (0 of 7) cases, respectively. No statistically significant correlation was identified among the gene amplification indices, the RNA expression scores, and protein expression scores; this result may be related in part to the great intratumoral heterogeneity of c-*myc* expression at both RNA and protein levels. In nearly all of the tumor samples, areas of tumor cells were observed that were both positive and negative for expression of the mRNA and proteins of c-*myc* gene, irrespective of the gene amplification status. No specific tumor histology was identified specifically for the positive expression nor the negative expression of c-*myc* at either RNA or protein level. Some areas of tumor cells that were negative for RNA expression were positive for the protein, and vice versa. Predominantly nuclear staining of c-Myc protein was found in stromal fibroblasts, in well-differentiated tumors, as well as in invasive tumor areas. However, predominantly cytoplasmic staining of c-Myc was observed only in the widely invasive tumor cells, not in fibroblasts, hyperplstic lesions, nor well-differentiated tumor areas. These results indicate that high-grade breast cancer may have high frequency but low copy number of the c-*myc* gene amplification, as well as high frequencies of overexpression at the RNA and protein levels. The gene amplification, RNA overexpression and protein overexpression of c-*myc* are not necessarily interrelated. The occurrence of predominantly cytoplasmic localization of c-Myc protein may correlate with the tumor aggressiveness and thus a poor prognosis.

5. Study of the relationships among cyclins and c-Myc in human breast cancer: In the c-*myc* transgenic mammary tumors, expression of c-*myc* was found to be colocalized with that of cyclin A2 but was reciprocally related to the expression of cyclins D1 and E. These results indicate that c-Myc might induce cyclin A2 but suppress cyclins D1 and E. We therefore further investigated whether such interrelationships among cyclins and c-Myc occurred also in human breast cancer (5). The results were summarized below:

Abstract (summary of appendix V):
Many *in vitro* studies have suggested that cyclins D1, E and A2 are putative target genes of c-Myc and their expression is regulated by c-Myc. However, few studies have addressed whether these c-Myc regulations really occur in a human cancer. Characterization of these issues may be helpful for the understanding of many paradoxical issues pertaining to the relationship between overexpression of these genes and many clinicopathologic parameters. In the present study, we examined the topographic relationships among the expression of these genes by comparison of the same patches of tumor cells immunohistochemically stained for c-Myc and these cyclins on serial sections. Four patterns of relationships between c-Myc and cyclin D1 were observed in different patches of tumor cells, i.e. negative c-Myc with nuclear cyclin D1, positive nuclear c-Myc with basically negative cyclin D1, strong cytoplasmic cyclin D1 with nuclear c-Myc, and nuclear c-Myc with both nuclear and cytoplasmic cyclin D1. Expression of cyclin E, no matter in the cytoplasm, both cytoplasm and nucleus or the nucleus, was always associated with expression of cyclinD1, c-Myc, or both. Both reciprocal and concomitant expression of cyclin E and cyclin D1 were observed in different patches of tumor cells. Expression of cyclin A2 did not seem to particularly be associated with any of the c-Myc and cyclins D1 and E. However, cyclin A2 labeling indices were statistically associated with the Ki67 labeling indices, indicating that expression of cyclin A2, in general, reflected the proliferation of tumor cells. Invasive tumor cells that were disseminated in the stromal or fat tissue usually manifested a concomitant staining of c-Myc and cyclins D1 and E, but not cyclin A2, in the nucleus. Taken together, these results suggest, indirectly, that c-Myc and cyclin D1 may usually be reciprocally expressed, whereas expression of cyclin E may require either cyclin D1 or c-Myc. Concomitant expression of c-Myc and cyclins D1 and E in the nucleus may reflect a more invasive potential than expression of each of these genes alone. Expression of cyclin A2 may reflect tumor cell proliferation in general, but it should be more cautious if it is used as a surrogate of proliferation marker.

KEY RESEARCH ACCOMPLISHMENTS TO DATE:

- We have found that c-*myc* mammary tumors develop "tumor-within-a-tumor" foci, which makes c-*myc* mouse a good animal model of mammary carcinogenesis that shows recognizable multistages of tumor progression.
- Our data suggest that in the mammary gland, c-Myc may induce E2F1 and cyclin A2 to initiate the tumor development, whereas overexpression of cyclins D1 and E may occur as later events to promote tumor progression to a more aggressive phenotype.

- Our data also suggest that TGFα may enhance c-Myc-induced mammary carcinogenesis by inducing cyclin D1 and facilitating the loss of pRB, resulting in an earlier development of more aggressive tumors, compared to the c-*myc* tumors.
- Our observations show that c-Myc-induced cell death in mammary tumors is not a typical apopotosis as one may assume based on studies with cultured cells.
- We have shown that in high-grade human breast cancer, the c-*myc* gene is frequently amplified, but the copies of gene gains are usually low. Cytoplasmic localization of overexpressed c-Myc protein may be associated with tumor aggressiveness.
- Our data suggest that in human breast cancer, concomitant expression of c-Myc and cyclins D1 and E may reflect a more invasive potential than the expression of each of these genes alone, whereas expression of cyclin A2 may reflect tumor cell proliferation.

REPORTABLE OUTCOMES FOR THIS PERIOD:

1. Published a paper in "Oncogene", which described the cell cycle profile in c-*myc* and *tgfα*/c-*myc* transgenic mammary tumors.
2. Published a review article in "Endocrine-Related Cancer", which described the literature and our hypothesis on the role of c-*myc* in breast cancer.
3. Submitted a manuscript to "Oncogene" to present data on the cell death properties of c-*myc* transgenic mammary tumors.
4. Submitted a manuscript to "Clinical Cancer Research" to present the c-*myc* amplification and overexpression in human breast cancer biopsies.
5. Complete a manuscript to be submitted to "American Journal of Pathology" to present our data on the relationships among cyclins and c-Myc.
6. Complete a review article for a publication in a special issue of "Journal of Steroid Biochemistry & Molecular Biology", which we are editing. The article summarizes the literature and our hypothesis on the roles of androgens in the mammary gland.

CONCLUSIONS:

During the first year of this award, we have accomplished tasks 1a, 1d, 2d, 3a, 3e, 4b and part of 4a; some of these tasks were originally scheduled for the second and third years. The results indicate that c-Myc may induce E2F1 and cyclin A2 to initiate the tumor development, whereas overexpression of cyclins D1 and E may occur as later events to promote tumor progression to a more aggressive phenotype. TGFα may enhance c-Myc-induced mammary carcinogenesis by inducing cyclin D1 and facilitating the loss of pRB expression. While accomplishing the above tasks, we have postponed tasks 1b, 1c 2a, 2b, and 2c as the future work, due to the unexpected finding of p53 mutations in Commad1 and HC14MV4 cells. Moreover, we have also accomplished several studies on c-Myc and cyclins in human breast cancer, and have written a review article on the sex hormones in the mammary gland (6). These lines of work were not scheduled in the experimental tasks, but were described as parts of the Career/Research Plans of this award.

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2. Liao, D-Z.J., and Dickson, R.B. (2000) c-Myc in breast cancer. Endocrine-Related Cancer, 7: 143-164.
3. Liao, D-Z. J., Chepko, G., and Dickson, R.B. (2001) Atypical apoptosis in MMTV-c-myc transgenic mouse mammary tumors. Submitted to Oncogene.
4. Blancato, J., Dickson, Fei, X-F., Liao, D-Z. J. (2001) Amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridization, and immunohistochemical analyses. Submitted to Clinical Cancer Res.
5. Liao, D-Z. J., Blancato, J., and Dickson, R.B. (2001) Topographic relationships among the expression of cyclins D1, E, and A2 and c-Myc in high-grade breast cancer. To be submitted to Am. J. Pathol.
6. Liao, D-Z.J. and Dickson, R.B. (2001) Roles of androgens in the development, growth, and carcinogenesis of the mammary gland. To be published in "Proliferation of Normal and Malignant Mammary Epithelial Cells"—A special issue of J. Steroid Biolchem. Mol. Biol (guess-edited by R.B. Dickson and D-Z. J. Liao)

APPENDICES:

Appendix I: Liao, D-Z. J., Natarajan, G., Deming, S.L., Jamerson, M.H., Johnson, M., Chepko, G., and Dickson, R.B. (2000) Cell cycle basis for the onset and progression of c-Myc-induced, TGFα-enhanced mouse mammary gland carcinogenesis. Oncogene, 19: 1307-1317.

Appendix II: Liao, D-Z.J., and Dickson, R.B. (2000) c-Myc in breast cancer. Endocrine-Related Cancer, 7: 143-164.

Appendix III: Liao, D-Z. J., Chepko, G., and Dickson, R.B. (2001) Atypical apoptosis in MMTV-c-myc transgenic mouse mammary tumors. Submitted to Oncogene.

Appendix IV: Blancato, J., Dickson, Fei, X-F., Liao, D-Z. J. (2001) Amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridization, and immunohistochemical analyses. Submitted to Clinical Cancer Res.

Appendix V: Liao, D-Z. J., Blancato, J., and Dickson, R.B. (2001) Topographic relationships among the expression of cyclins D1, E, and A2 and c-Myc in high-grade breast cancer. To be submitted to Am. J. Pathol.

Appendix VI: Liao, D-Z.J. and Dickson, R.B. (2001) Roles of androgens in the development, growth, and carcinogenesis of the mammary gland. To be published in "Proliferation of Normal and Malignant Mammary Epithelial Cells"—A special issue of J. Steroid Biolchem. Mol. Biol (guess-edited by R.B. Dickson and D-Z. J. Liao)

Appendix I

Oncogene (2000) 19, 1307–1317

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Cell cycle basis for the onset and progression of c-Myc-induced, TGFα-enhanced mouse mammary gland carcinogenesis

Dezhong J Liao[1], Geraldine Natarajan[1], Sandra L Deming[1], M Hunter Jamerson[1], Michael Johnson[1,2,3], Gloria Chepko[1] and Robert B Dickson*[,1,3,4]

[1]*Vincent T Lombardi Cancer Center, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA;* [2]*Department of Pathology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA;* [3]*The Department of Oncology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA;* [4]*Department of Cell Biology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA*

Using single and double transgenic mouse models, we investigated how c-Myc modulates the mammary epithelial cell cycle to induce cancer and how TGFα enhanced the process. In c-*myc* transgenic mice, c-*myc* expression was high in the hyperplastic mammary epithelium and in the majority of tumor areas. However, the tumors displayed focal areas of low expression of c-*myc* but high rates of proliferation. In contrast to E2F1 and cyclin A2, which were induced and co-localized with c-*myc* expression, induction of cyclins D1 and E occurred only in these tumor foci. Overexpression of cyclin D1 also occurred in the hyperplastic epithelium of *tgfα*-single and *tgfα*/c-*myc*-double transgenic mice. In *tgfα*/c-*myc* tumors, cells positive for cyclins D1 and E were randomly spread, without showing a reciprocal relationship to c-*myc* expression. In contrast to c-*myc* tumors, most *tgfα*/c-*myc* tumors showed undetectable levels of retinoblastoma protein (pRB), and the loss of pRB occurred in some cases at the mRNA level. These results suggest that E2F1 and cyclin A2 may be induced by c-Myc to mediate the onset of mammary cancer, whereas overexpression of cyclins D1 and E may occur later to facilitate tumor progression. TGFα may play its synergistic role, at least in part, by inducing cyclin D1 and facilitating the loss of pRB. *Oncogene* (2000) **19,** 1307–1317.

Keywords: c-Myc; TGFα; E2F; cyclins; pRB; cell cycle

Introduction

The c-Myc protein plays a crucial role in cell proliferation, differentiation, apoptosis, and transformation (Schmidt, 1999; Facchini *et al.*, 1998; Amati *et al.*, 1998; Dang, 1999). Overexpression, amplification, or rearrangement of the c-*myc* gene has been reported in over 50% of human breast cancer cases (Nass *et al.*, 1997; Amundadottir *et al.*, 1996a). About half of the virgin female mice carrying the c-*myc* transgene under control of mouse mammary tumor virus (MMTV) long terminal repeat also develop spontaneous mammary carcinomas after 9 months of age (Stewart *et al.*, 1984; Amundadottir *et al.*, 1995, 1996b). c-Myc-induced carcinogenesis may be further promoted by additional growth stimuli such as some female sex hormones, since multiple pregnancies markedly increase its incidence and shorten its latency period (Stewart *et al.*, 1984; Amundadottir *et al.*, 1995, 1996b).

One major mechanism for c-Myc to exert its functions involves its action as a transcription factor, heterodimerizing with Max and binding to the Myc E-box elements of its target genes (Cole *et al.*, 1999). Thus, cdc25A and cyclins E and A2 have been suggested as direct, c-Myc-activated target genes (Cole *et al.*, 1999; Obaya *et al.*, 1999). In contrast, the relationship between c-Myc and cyclin D1 is still under debate in the literature (Facchini *et al.*, 1998; Dang, 1999). The 5′-flanking region of the cyclin D1 gene in mouse and human contains a c-Myc recognition site (Daksis *et al.*, 1994), and expression of cyclin D1 has been shown to be induced in some c-*myc*-expressing tumor cells (Facchini *et al.*, 1998; Dang, 1999), in liver tissue, and in liver tumors from mice carrying a c-*myc* transgene under the control of the albumin gene promoter (Santoni-Rugiu *et al.*, 1998). These data seem to suggest that cyclin D1 may be a direct target of activation by c-Myc. However, it has also been shown in other systems that c-Myc suppresses transcription of cyclin D1 (Philipp *et al.*, 1994; Jansen-Durr *et al.*, 1993; Marhin *et al.*, 1996). Still other studies suggest that cyclin D1 is not a target of c-Myc-signaling but represents a pathway parallel to c-Myc signaling for control of cell replication (Roussel, 1998; Bodrug *et al.*, 1994; Alexandrow *et al.*, 1998; Solomon *et al.*, 1995). Nevertheless, these four putative c-Myc targets (cdc25A, cyclins E, A2 and D1) can function to activate cyclin dependent kinases (cdk) 4, 6 or 2 during G1 and S phases, resulting in phosphorylation of the retinoblastoma protein (pRB). pRB-associated transcription factors, of which E2F1 is the most important, are thus released and activated (Morgan, 1995; Sherr, 1996). Free E2F1 activates transcription of genes required for S phase entry and progression (Johnson *et al.*, 1998; Lavia *et al.*, 1999).

Voluminous literature has causally connected cancer onset and progression to abnormal expression or gene structure (amplification or mutation) of cyclins D1, E, and A2, as well as cdk inhibitors p16, p27, and p21 (or its key regulator, p53) (Morgan, 1995; Sherr, 1996; Gray-Bablin *et al.*, 1996; Keyomarsi *et al.*, 1993; Steeg *et al.*, 1998; Barnes *et al.*, 1998). Each of these genes encodes a protein controlling a step(s) along the cyclin-cdk-pRB pathway, alteration in which presumably results in an increase in free, active E2F1 or other

*Correspondence: RB Dickson, Research Building, W417, 3970 Reservoir Road NW, Lombardi Cancer Center, Georgetown University Medical Center, Washington DC 20007, USA
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E2F family members. This implies that E2F1 plays a central role in cancer development (Johnson *et al.*, 1998). Overexpression of E2F1 is an intriguing mechanism for its activation in the context of c-Myc-induced carcinogenesis, since E2F1 expression has been shown to be induced in liver from c-*myc* transgenic mice (Santoni-Rugiu *et al.*, 1998) and in fibroblasts transfected with the c-*myc* gene (Leone *et al.*, 1997).

Transforming growth factor α (TGFα) is a strong mitogen for a variety of cell types (Lee *et al.*, 1995; Dickson *et al.*, 1995) and is overexpressed in over 50% of breast cancer cases (Auvinen *et al.*, 1996; Pilichowska *et al.*, 1997; Panico *et al.*, 1996). Virgin female mice carrying a *tgfα* transgene under control of the MMTV or metallothionein-1 (MT) promoters develop mammary epithelial hyperplasia, but not mammary cancer, unless the mice undergo multiple pregnancies (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990; Matsui *et al.*, 1990). However, dual carriers of c-*myc* and *tgfα* transgenes, generated in our laboratory by mating the MMTV-c-*myc* strain to the MT-*tgfα* strain, develop mammary cancers at 100% incidence, in both females and males, soon after 2 months of age. In addition, the tumors grow much faster than those occurring in the c-*myc* single transgenic strain (Amundadottir *et al.*, 1995, 1996b). These data demonstrate that TGFα overexpression strikingly enhances c-Myc-induced carcinogenesis (Sinn *et al.*, 1987), in line with the *in vitro* studies showing that co-transfection of cells with *tgfα* and c-*myc* effectively induces transformed phenotype, in contrast to transfection of either gene alone (Amati *et al.*, 1998; Land *et al.*, 1983). The mechanisms for this synergistic influence of TGFα are not yet fully clarified. With respect to the interactions of these two proteins at the cell cycle level, one possibility is that the synergistic role of TGFα is exerted via the Ras/Raf cascade, a major TGFα signaling pathway (Lee *et al.*, 1995), since overexpression of c-Ras^H has been shown to increase the c-Myc protein levels (Kerkhoff *et al.*, 1998; Sears *et al.*, 1999). Also, co-expression of c-Myc and activated c-Ras^H, but not either gene alone, is able to transform cells in culture (Amati *et al.*, 1998; Land *et al.*, 1983). However, the Ras/Raf pathway seems to recruit cyclin D1 as a major step (Lukas *et al.*, 1996), whereas synergy between c-Ras^H and c-Myc has been shown in fibroblasts to be elicited via induction of E2F1 and activation of cyclin E-cdk2, without affecting either cyclin D1 activity or pRB phosphorylation (Leone *et al.*, 1997).

By using three transgenic mouse models, in this study we set out to explore the cell cycle regulatory mechanisms whereby c-Myc elicits mouse mammary tumors and to determine how TGFα synergistically modulates these mechanisms. We found that in c-*myc* transgenic mice, induction of cyclin A2 and E2F1 were most closely associated with expression of the c-*myc* transgene and might thus mediate tumor onset. In contrast, overexpression of cyclins D1 and E occurred as later events in morphologically distinctive, rapidly growing, poorly apoptotic foci within established c-*myc* tumors. In our synergistic, bi-transgenic model, TGFα appeared to immediately induce cyclin D1 and to cooperate with c-Myc to attenuate the levels of pRB protein. We propose that these two TGFα-mediated effects may be associated with the earlier onset and faster growth of the mammary cancer in the bi-transgenic model.

Results

Morphologic characteristics of mammary tumors

In MT-*tgfα* transgenic mice, mammary glands showed hyperplasia, but without tumor formation. As observed also by others (for review see Cardiff *et al.*, 1995), the mammary tissue contained abundant, proliferating stroma. In marked contrast, the hyperplastic mammary tissue from MMTV-c-*myc* animals did not show pronounced stromal proliferation. Stromal cells were also abundant in hyperplastic mammary tissue and mammary carcinomas from bi-transgenic *tgfα*/c-*myc* mice. The epithelial cells in non-tumor areas of the mammary glands from bi-transgenic mice usually manifested atypical hyperplastic features that were similar to the morphology of tumor cells. Thus, there was no clear-cut evidence for pre-malignant stages of this tumor type.

In c-*myc* transgenic animals, about half of the relatively larger (1 cm or larger in diameter) tumors

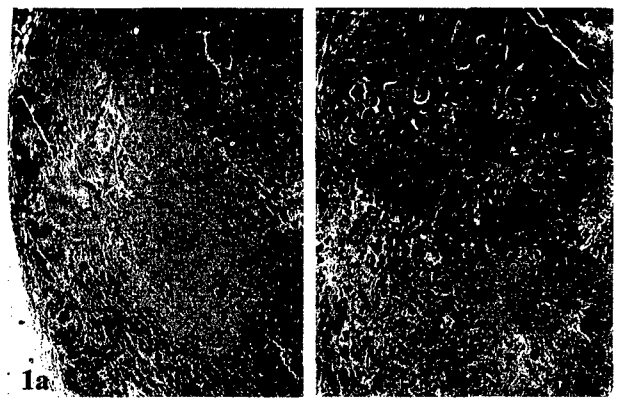

Figure 1 Hematoxylin-eosin staining of mammary tumors from two c-*myc* animals, showing three individual foci (F1, F2 and F3) within the tumors. Some areas of the foci show infiltrating growth into the adjacent tumor areas (arrow). Necrosis (N) can be discerned in focus 2

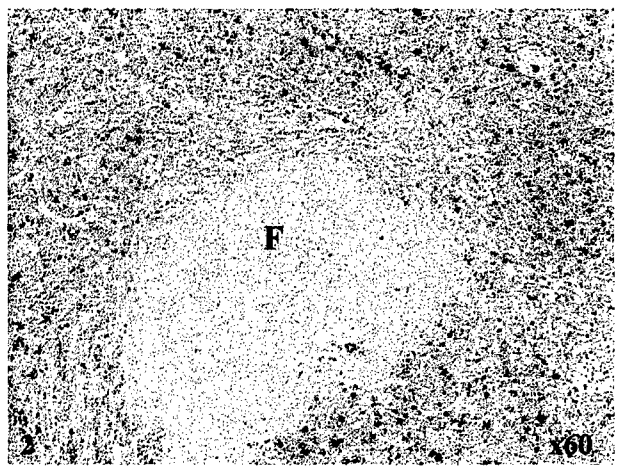

Figure 2 Tunel staining of a tumor from a c-*myc* animal, showing that apoptotic cells (dark brown staining) are distributed predominantly in the major tumor area, but rarely in the focus

contained foci that consisted of tumor cells with distinctive morphology. Specifically, tumor cells within the foci were characterized by larger nuclei and weaker staining for hematoxylin and eosin (Figure 1a,b). Although this 'tumor within a tumor' showed a clear boundary of demarcation from surrounding tumor areas, it was not encompassed by a connective tissue capsule. Usually, some portion of each focus exhibited

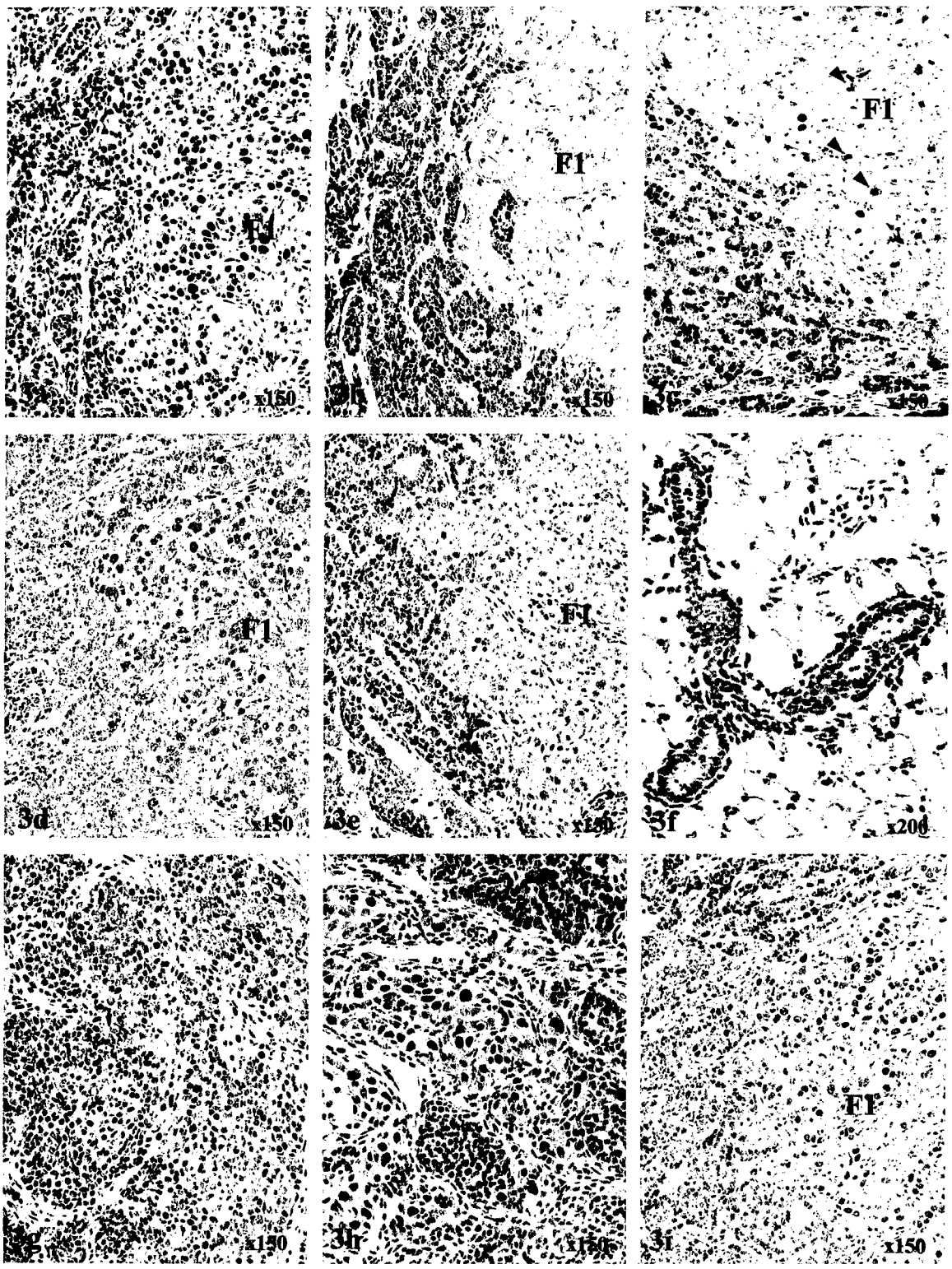

Figure 3 Immunohistochemical staining (brown color) with light hematoxylin counter-staining (blue color). Staining shown in (**a**–**e**) and (**i**) was carried out on serial sections of the same tumor and tumor focus (F1) as shown in Figure 1 (**a**). Tumor cells inside focus 1 show stronger staining for PCNA than cells outside the focus (**a**). Most tumor cells outside, but not inside, the focus exhibit strong staining for c-Myc (**b**) and cyclin A (**c**), although some stromal cells inside the focus are also positive for cyclin A (arrows). Conversely, most tumor cells inside the focus exhibit strong staining for cyclin D1 (**d**) and cyclin E (**e**), while tumor cells outside the focus are negative. Many cells in the hyperplastic mammary gland from a *tgfα* animal (**f**) and in a tumor from a *tgfα*/c-*myc* animal (**g**) also show strong cyclin D1 staining. In a c-*myc* tumor, some cyclin E-positive cells show a trend of penetrating (from up-left side) into the adjacent, cyclin E-negative area (low-right side) (**h**). Staining for cdk4 (**i**) is more intense in many tumor cells inside the focus than those in the adjacent area

infiltration into the adjacent, surrounding tumor areas (Figure 1). Necrotic areas were occasionally seen inside the foci (Figure 1b). Very strikingly, apoptotic cells within each focus were much less frequent than in the surrounding tumor areas. When foci were observed, their numbers varied between two and four in each random cross-section and their sizes varied from microscopic to about 3 mm in diameter for the animal ages of 10–12 months. The foci were not seen in tumors less than 1 cm in diameter, indicating that they might have occurred selectively at relatively advanced progression states. No such specific foci were observed in tumors from *tgfα*/c-*myc* double transgenic mice.

Assessment of cell proliferation and apoptosis

In c-*myc* tumors, PCNA staining was more intense in the specific foci than in their surrounding tumor areas (Figure 3a). The staining in *tgfα*/c-*myc* tumors was as intense as in the c-*myc* tumor foci. Moreover, the staining index for the c-*myc* tumor foci (39.1% ± 3.4) was higher than that for their surrounding tumor tissue (20.4% ± 4.0, $P<0.01$), but it was comparable to that for *tgfα*/c-*myc* tumors (44.4% ± 4.2, $P>0.05$). Hyperplastic mammary glands from all three lines of transgenic animals also showed some strongly stained cells, but the fraction was too small to allow calculation of a reliable index. PCNA-positive cells were not observed in mammary glands from the normal, non-transgenic animals.

In contrast to the PCNA staining results, the TUNEL assay for apoptotic cells showed a much higher labeling index in the major areas (15.8% ± 1.8) than in the foci (1.0% ± 1.1, $P<0.01$) of c-*myc* tumors (Figure 2). The TUNEL labeling index in the *tgfα*/c-*myc* tumors (1.7% ± 1.1) was comparable to that in the foci of c-*myc* tumors ($P>0.05$).

*Expression of c-*myc

Consistent with the data reported previously (Amundadottir *et al.*, 1995), c-*myc* mRNA was abundantly expressed in hyperplastic mammary epithelium (Figure 4a) and in tumors (Figure 4b) from c-*myc* mice, but was undetectable in normal mammary tissue from age-matched, non-transgenic animals. A sense probe did

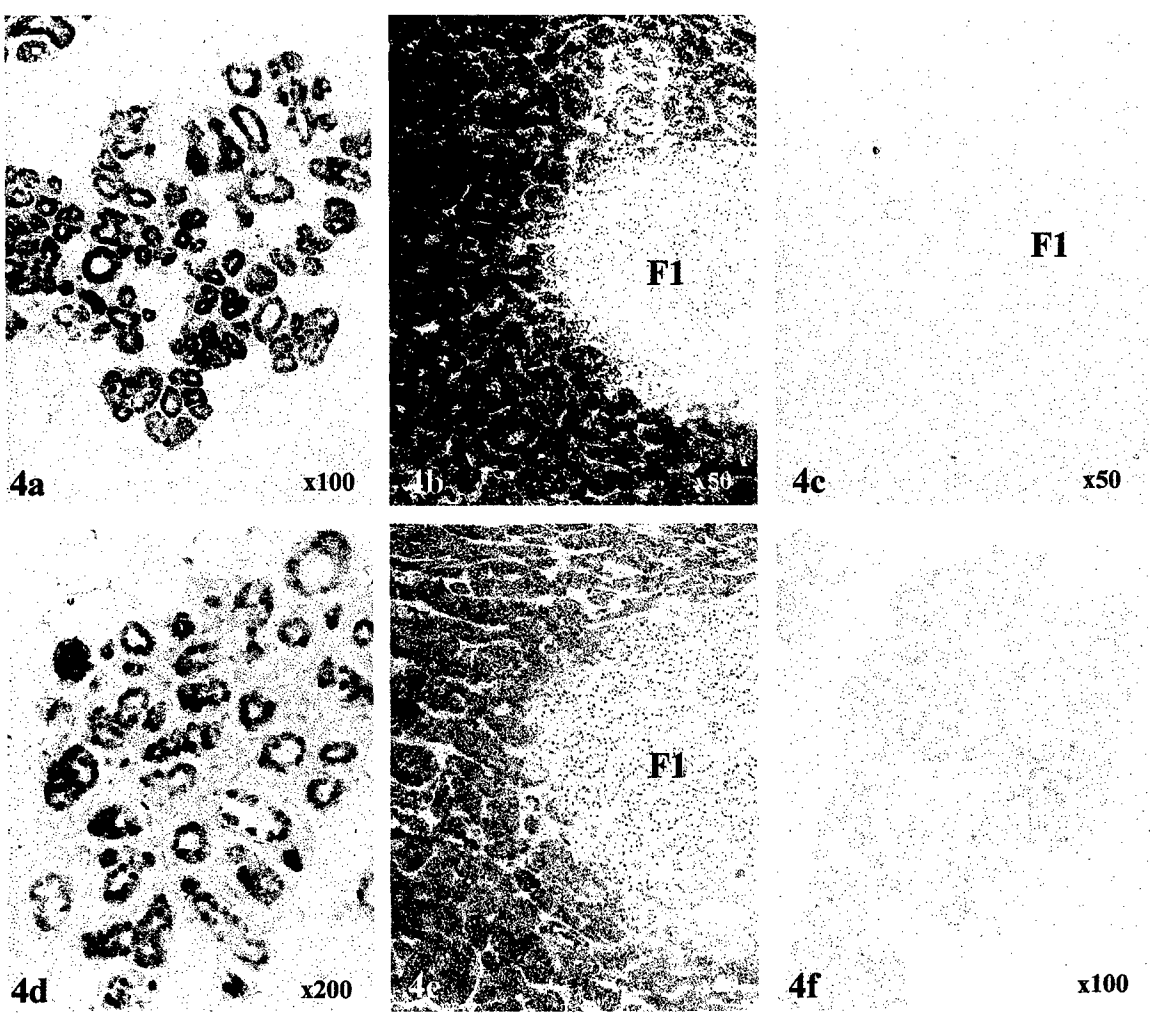

Figure 4 Nonradioactive *in situ* hybridization for c-*myc* (**a**–**c**) and *e2f1* (**d**–**f**). Hybridizations shown in (**b**), (**c**), and (**e**) were carried out on serial sections of the same tumor and tumor focus (F1) as shown in Figure 1 (**a**). In c-*myc* animals, high levels of c-*myc* mRNA were detected by antisense probe in hyperplastic mammary glands (**a**) and in the major tumor area, but not the tumor focus (**b**). No signal was detected in the same tumor area when a sense probe was used (**c**). High levels of *e2f1* mRNA expression were detected by an antisense probe (**d**) in hyperplastic mammary glands from a c-*myc* animal. The *e2f1* mRNA expression co-localizes with c-*myc* expression in the same tumor as shown in 2(**b**) (**e**). No signal was detected in the hyperplastic mammary glands from c-*myc* animal when an *e2f1* sense probe was used (**f**)

not give rise to a signal in any of these tissues (Figure 4c), demonstrating that the signal detected by the antisense probe is specific for the c-*myc* mRNA. The foci in c-*myc* tumors showed very low levels of its expression (Figure 4b), in strong contrast to their adjacent areas with high levels of c-*myc* mRNA. Immunohistochemical results also showed a much stronger positive staining in the major tumor areas than in the foci (Figure 3b). Western blot analyses revealed much higher levels of c-Myc protein in mammary tumors, compared to hyperplastic mammary tissue (Figure 5). However, this difference was due largely to the heterogeneity in cellularity, as the mammary tissues used for protein sample preparation were fat pads dominated by fat tissue. Hyperplastic epithelium and tumors from *tgfα*/c-*myc* animals also expressed high levels of c-*myc* mRNA and protein, while expression of c-*myc* was not detected in the hyperplastic epithelium from *tgfα* animals.

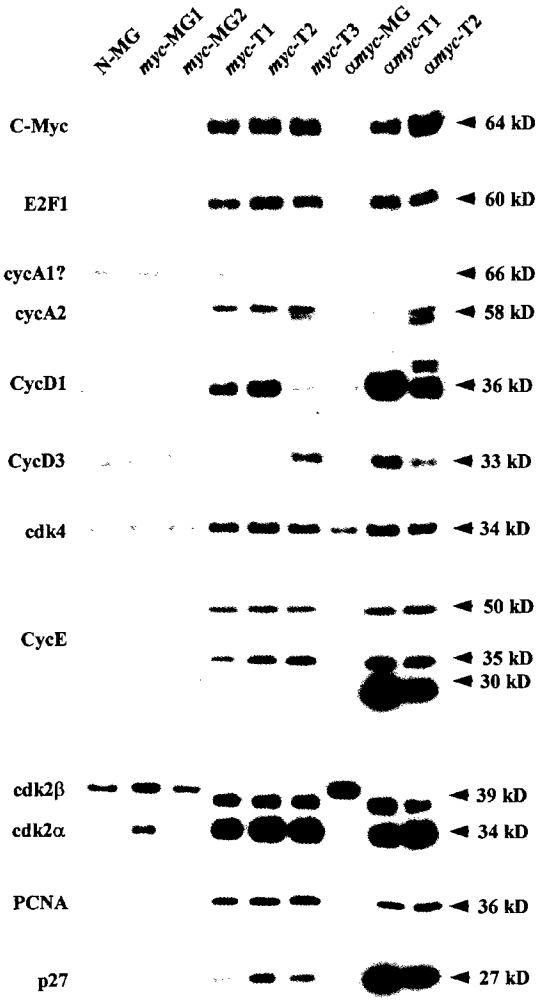

Figure 5 Western blot analyses for size comparisons of various proteins. N-MG: protein samples from normal mammary tissue pooled equally from three nontransgenic animals; *myc*-MG: hyperplastic mammary tissue from two individual c-*myc* animals; *myc*-T: three representative mammary tumors from c-*myc* animals; *αmyc*-MG: hyperplastic mammary gland tissue pooled from two individual *tgfα*/c-*myc* animals; *αmyc*-T: two representative tumors from *tgfα*/c-*myc* animals. Quantitative comparisons among different samples may not be made, as tumor tissues enriched in protein whereas non-tumor tissues were dominated by protein-poor fat tissue

Expression of E2F1

An *e2f1* antisense probe detected strong signals in the hyperplastic mammary epithelium, from both c-*myc* (Figure 4d) and *tgfα*/c-*myc* animals, but not in that from *tgfα* transgenic mice, suggesting that the induction of *e2f1* mRNA was specifically related to expression of c-Myc, but not TGFα. In tumors from c-*myc* animals, the major areas with high levels of c-*myc* mRNA and protein also expressed high levels of *e2f1*, whereas those specific tumor foci with low expression levels of c-*myc* exhibited very low levels of *e2f1* (Figure 4e), indicating that expression of *e2f1* and c-*myc* are co-localized. High levels of *e2f1* expression were also detected in *tgfα*/c-*myc* tumors (Table 1). The sense probe did not give rise to signal (Figure 4f). Northern blot analysis detected the expected two *e2f1* transcripts (Li *et al*., 1994) in c-*myc* and *tgfα*/c-*myc* tumors (Figure 6). Western blot analysis also confirmed high levels of the E2F1 protein in these tumors (Figure 5). Immunohistochemical staining on paraffin-embedded tissues was not successful with this, nor with other antibodies.

Expression of cyclin A2

Immunohistochemistry for cyclin A2 showed that in c-*myc* tumors, positive tumor cells were localized mainly to the major areas with high levels of c-Myc (Figure 3c), indicating that expression of cyclin A2 and c-*myc* may be co-localized. Many positive cells were also discerned in the hyperplastic epithelium from c-*myc* animals, as well as in the atypical hyperplastic epithelium and tumors from *tgfα*/c-*myc* mice, but not in the epithelium

Table 1 Relationship among expression of c-Myc and of cell cycle components in c-*myc* and *tgfα*/c-*myc* tumors

	c-myc *tumors*		tgfa/c-myc
	Tumor cells with high levels of c-Myc	*Focal tumor cells with low levels of c-Myc*	*tumor with high levels of c-Myc*
E2F1	+++*	–	+++
Cyclin A	++	–	++
Cyclin D1	–	+++	+++
Cyclin E	–	+++	+++
Cdk2	++	++	++
Cdk4	+	+++	+++
Cyclin D3	+	+++	+++
PCNA	+	+++	+++
P16	+	+	+
P21	++	++	++
P27	++	++	+++

*Expression levels of the genes are subjectively grouped from three '+' to '–' in order from the strongest positive to the most negative

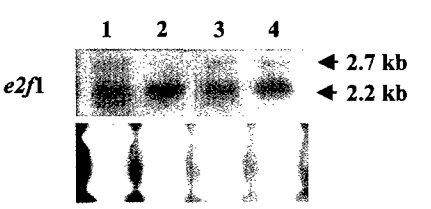

Figure 6 Northern blot analysis of the *e2f1* gene, demonstrating that *e2f1* mRNA was expressed in two randomly selected tumors from c-*myc* animals (1 and 2) and *tgfα*/c-*myc* animals (3 and 4). Loading of total RNA (10 μg per lane) was visualized by ethidium bromide staining of the gel (lower panel)

from *tgfα* animals (data not shown). On Western blot, cyclin A2 protein, at about (~) 58 kD was detected in c-*myc* and *tgfα*/c-*myc* tumors (Table 1), but not in hyperplastic mammary tissues (Figure 5). The antibody also recognized a protein at ~66 kD that, in contrast, was mainly present in normal and hyperplastic mammary tissue. This protein is likely to be cyclin A1, a newly identified member of the cyclin A family (Sweeney *et al.*, 1996; Yang *et al.*, 1997), as the peptide used for generating the antibody differs from the corresponding sequence of cyclin A1 by only a few amino acid residues. This protein might contribute to the immunohistochemical staining of some stromal cells in the major tumor areas and in the foci of c-*myc* tumors (Figure 3c, arrows), since similarly positive cells were also observed in normal mammary tissue from non-transgenic mice in which only the ~66 kD protein was detected by Western blot (Figure 5).

Expression of cyclin D1

In c-*myc* animals, cyclin D1-positive cells could be observed only in tumors, not in hyperplastic mammary epithelium. In the tumors, the cyclin D1-positive cells were exclusively localized to the specific foci with low levels of c-Myc, but not in the major areas with high levels of c-Myc (Figure 3d), suggesting that expression of cyclin D1 and c-Myc may be reciprocal. In contrast to the c-*myc* tumors, a large number of tumor cells in *tgfα*/c-*myc* animals manifested strong staining of cyclin D1 (Table 1), and they were randomly spread within the whole tumor, without forming any specific focus, nor showing reciprocal expression to c-*myc* (Figure 3g). Many cells in the hyperplastic mammary epithelium from *tgfα* (Figure 3f) and *tgfα*/c-*myc* mice also exhibited strong staining of cyclin D1, indicating that cyclin D1 expression might be induced by TGFα in the epithelium prior to tumor formation. Western blot analysis confirmed the presence of high levels of cyclin D1 protein in tumors from c-*myc* and *tgfα*/c-*myc* mice (Figure 5).

Expression of cyclin E

In c-*myc* tumors, cyclin E-positive cells were found to be co-localized with cyclin D1, exclusively in the specific focal lesions, but not the major areas (Figure 3e). Moreover, cyclin E-positive cells usually showed a trend for penetration into the adjacent areas (Figure 3h), indicating that they might have a stronger invasive potential. In *tgfα*/c-*myc* tumors, cyclin E-positive cells were randomly spread throughout the whole tumor (Table 1), without forming specific foci, similar to the distribution of cyclin D1-positive cells. However, at the subcellular level, the cyclin E staining was localized in both nucleus and cytoplasm, unlike the solely nuclear staining seen in c-*myc* tumors. Hyperplastic epithelium from *tgfα* and *tgfα*/c-*myc* mice was negative or weakly positive for cyclin E in some cells, indicating that cyclin E was not significantly induced by TGFα alone.

On Western blot (Figure 5), cyclin E proteins in c-*myc* and *tgfα*/c-*myc* tumors were present, not only as the full-length form of ~50 kD, but also as several smaller isoforms, as reported by others for breast cancer tissue and for cell lines derived from human and mouse (Gray-Bablin *et al.*, 1996; Keyomarsi *et al.*, 1993; Said *et al.*, 1995; Sgambato *et al.*, 1996). Interestingly, an ~28 kD, putative cyclin E protein was the dominant isoform in *tgfα*/c-*myc* tumors; this isoform was barely discernible in c-*myc* tumors. This cyclin E isoform may thus account for the cytoplasmic staining seen in *tgfα*/c-*myc* tumor cells.

Expression of cdk4, cyclin D3, and cdk2

Many cells in the mammary epithelium from non-transgenic mice and from c-*myc*, *tgfα*, and *tgfα*/c-*myc* animals were positive for cdk4 by immunohistochemical staining. In c-*myc* tumors, cdk4 positive cells were observed both in the cyclin D1-positive foci and in the major areas that were cyclin D1-negative, but the staining intensity was stronger in many cells within the foci (Figure 3i). No obvious differences in the staining were observed between c-*myc* tumors and *tgfα*/c-*myc* tumors (Table 1). Similar immunohistochemical data were obtained for cyclin D3 (Table 1). Western blot analyses also detected the cdk4 and cyclin D3 proteins in these tumors and in mammary tissues from non-transgenic or various transgenic animals (Figure 5).

Immunohistochemical staining for cdk2 did not reveal differences among various mammary tissues and tumors. In c-*myc* tumors, both the major areas and the specific foci showed similar staining intensity (Table 1). Western blot assay for cdk2 detected both cdk2α at ~34 kD and cdk2β at ~39 kD, respectively (Kwon *et al.*, 1998; Kotani *et al.*, 1995; Noguchi *et al.*, 1993). In mouse, rat and hamster, the cdk2β is an alternate RNA splicing form of cdk2α, the classic cdk2, with an insert of 48 amino acids between amino acids 196 and 197 of cdk2α. The cdk2α isoform occurred as a single band in normal and hyperplastic mammary tissue, as well as in tumors, and was thus likely to be the inactivated, unphosphorylated form (Gu *et al.*, 1992; Planas-Silva *et al.*, 1997). Cdk2β, on the other hand, was present mainly as the phosphorylated, activated, faster-migrating form (Gu *et al.*, 1992; Planas-Silva *et al.*, 1997) in c-*myc* and *tgfα*/c-*myc* tumors, but it occurred mainly as the inactivated, unphosphorylated slower-migrating band in normal and hyperplastic mammary tissues (Figure 5).

Expression of cdk inhibitors

Immunohistochemical staining for p16 and p21 did not show pronounced differences between c-*myc* tumors and *tgfα*/c-*myc* tumors, and between the tumor foci and their surrounding areas in the c-*myc* tumors (Table 1). Western blot analyses of these two cdk inhibitors did not detect differences between c-*myc* tumors and *tgfα*/c-*myc* tumors (data not shown). However, the levels of p27 were higher in *tgfα*/c-*myc* tumors than in c-*myc* tumors, as measured by both immunohistochemical and Western blot analyses (Table 1 and Figure 5).

Expression of pRB protein

Protein levels of pRB varied among tumor samples but they were generally higher in c-*myc* than in *tgfα*/c-*myc* tumors; pRB levels in some representative samples are shown in Figure 7. The pRB protein was detectable by Western blot in all ten c-*myc* tumors studied; however, it was present in only two of eight *tgfα*/c-*myc* tumors.

pRB was present mainly as the hypophosphorylated form (Ezhevsky *et al.*, 1997). The faster-migrating, unphosphorylated band (c-*myc* tumor samples 1 and 4 in Figure 6) and its slower-migrating, hyperphosphorylated band of pRB (c-*myc* tumor samples 1 and 2 in Figure 6) could be discerned faintly in some samples, when the autography was exposed for such a short time that signals could not be detected in other samples. Two different pRB monoclonal antibodies (pRB14001A and pRB245) gave the same results by Western blot. However, immunohistochemical staining was not successful with either of these antibodies.

RT–PCR analysis was carried out for four *tgfα*/c-*myc* tumors and for three c-*myc* tumors, where a sufficient amount of tissue was available for RNA preparation. As shown in Figure 8, the Rb cDNA was detected in all three c-*myc* tumors and in the two *tgfα*/c-*myc* tumors that also showed detectable levels of pRB protein (Figure 7, samples 2 and 5), but not in the other two *tgfα*/c-*myc* tumors. The failure of the cDNA amplification in these two tumors was not due to a technical problem, since GAPDH cDNA, included as an internal control, was amplified normally (Figure 8).

Discussion

In this study we show that in c-*myc* transgenic mice, expression of cyclin A2 and E2F1 co-localizes with that of c-*myc* in hyperplastic mammary gland and in primary mammary tumors. Thus, we propose that these two genes may be induced either directly or indirectly by c-Myc to mediate the tumor onset. In support of this hypothesis, overexpression of cyclin A2 or E2F1 has been shown to directly facilitate transformation of cultured cells and to cause tumorigenesis in animals (Desdouets *et al.*, 1995; Amati *et al.*, 1998). Overexpression of each of these proteins has been reported in the pre-malignant liver tissue and spontaneous liver tumors in c-*myc* transgenic mice (Santoni-Rugiu *et al.*, 1998). Transfection of fibroblasts with c-*myc* has also been shown to induce *e2f1*, which is independent of pRB phosphorylation (Leone *et al.*, 1997), indicating that this effect may result directly from increased E2F1 protein, a short-cut mechanism that bypasses the cyclin-cdk-pRB pathway. In addition, since in c-*myc* tumors pRB is mainly in the hypophosphorylated state and presumably binds to and inactivates a portion of increased E2F1, cyclin A2 may be a more active element than E2F1 in cell proliferation and transformation. The short-cut mechanism and the rise of cyclin A2, which acts later in the cell cycle than cyclins D1 and E, may partly explain why overexpression of cyclins D1 and E does not occur in the majority of hyperplasia and primary tumor cells.

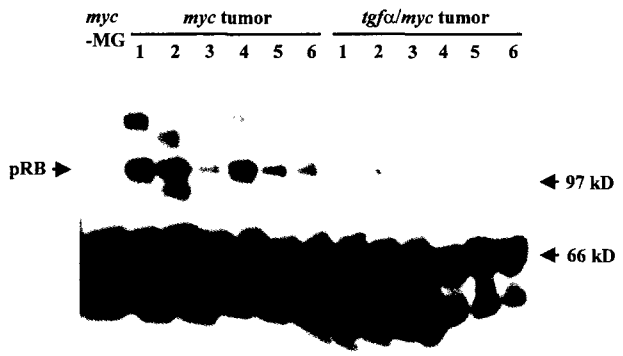

Figure 7 Western blot analysis of pRB. Eighty μg protein samples from hyperplastic mammary tissue were pooled equally from three individual c-*myc* animals (*myc*-MG) and from six individual c-*myc* tumors or *tgfα*/c-*myc* tumors and were loaded into the gel. Levels of the pRB at ~110 kD were generally higher in c-*myc* tumors than in *tgfα*/c-*myc* tumors. Two additional proteins at ~66 kD and ~55 kD were also recognized by (pRB14001A), levels of which were also slightly lower in some *tgfα*/c-*myc* tumors than in c-*myc* tumors

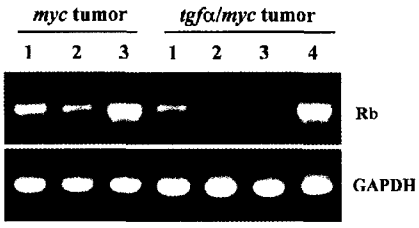

Figure 8 RT–PCR analysis of expression of Rb mRNA and GAPDH. Total RNA samples from three c-*myc* tumors and four *tgfα*/c-*myc* tumors were reverse-transcribed (RT), and the cDNA products were amplified by PCR, using the second pair of the primers described in Materials and methods. Note that two *tgfα*/c-*myc* tumors lack detectable expression of the Rb mRNA, while the GAPDH mRNA was expressed normally

The observation of a reciprocal expression of c-*myc* and cyclin D1 in c-*myc* tumors is the first evidence *in vivo* that favors, but does not prove, the concept that constant expression of c-Myc may suppress expression of cyclin D1. Several studies have shown that stable expression of cyclin D1, such as in mammary epithelial cells, paradoxically shortens the G1 phase and prolongs the S phase, while inhibiting growth and transformation to a malignant phenotype as the net consequence (Han *et al.*, 1995; Quelle *et al.*, 1993; Philipp *et al.*, 1994). Thus, it cannot be excluded that c-Myc suppresses expression of cyclin D1 in order to ensure a quicker completion of the cell division cycle and a more rapid onset of malignant transformation (Marhin *et al.*, 1996). However, once a tumor is formed, overexpression of cyclin D1 may be of selective value for its further progression to more aggressive phenotypes; a drop in the level of c-Myc could potentially facilitate release of repression of cyclin D1, while simultaneously decreasing c-Myc-induced apoptosis. This conjecture is supported by the decreased TUNEL labeling index in the progressed foci within primary c-*myc* tumors. This progression hypothesis (Figure 9) may explain why cyclin D1-positive cells are not seen in the hyperplastic lesions and in small tumors, but instead they occur in the foci within established c-*myc* tumors in association with enhanced staining of PCNA.

It should be noted that in human breast cancer samples, cyclin D1 overexpression is associated with immunohistochemical positivity for estrogen receptor (ER) (Barnes *et al.*, 1998). Interestingly, although ER positivity is, in general, considered a good prognostic marker, those ER positive cells that concomitantly overexpress cyclin D1 can continue to proliferate in the presence of anti-estrogens (Wilcken *et al.*, 1997). Although initially paradoxical, this is now not surprising, as cyclin D1 is known to form a direct complex with ER, allowing the complex to activate transcription without the need for estrogen (Neuman *et*

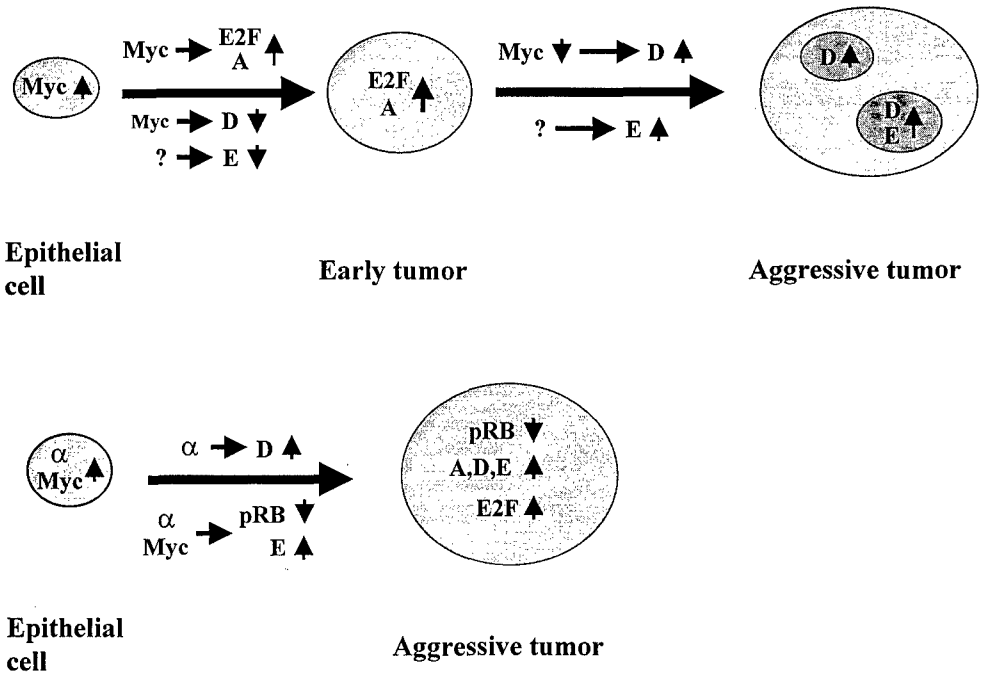

Figure 9 Illustration of hypothesis. In c-*myc* transgenic mice, constant overexpression of c-Myc protein in mammary epithelial cells directly or indirectly induces accumulation of E2F1 and cyclin A2 (A) to mediate tumor onset (upper panel). While the developing tumor continues to progress, a decrease in c-Myc expression occurs in some tumor cells, resulting in decreased apoptosis and in the overexpression of cyclin D1 (D). Cyclin E (E) overexpression is also triggered through an unknown mechanism. Each of these specific tumor cells then proliferates more aggressively to form a focus with distinct morphology. In the epithelial cells from *tgfα*/c-*myc* dual transgenic mice (lower panel), TGFα (α) induces overexpression of cyclin D1 and cooperates with c-Myc to induce overexpression of cyclin E and sporadic loss of pRB. These effects, together with the c-Myc-induced elevation of E2F1 and cyclin A2, elicit early onset of very aggressive tumor phenotypes in the bi-transgenic model

al., 1997). These observations, together with our progression hypothesis, may partly explain why about one-third of the ER-positive cases are refractory to antiestrogen therapy, why most of those who originally respond to antiestrogen later develop antiestrogen resistance (Lykkesfeldt, 1996), and why amplification of *cyclin D1* is associated with early relapse in patients with ER-positive breast cancer (Seshadri *et al.*, 1996).

In contrast to c-*myc* animals, in *tgfα* and *tgfα*/c-*myc* mice, overexpression of cyclin D1 is initially observed in the atypical hyperplastic mammary epithelium, indicating that cyclin D1 is induced by TGFα prior to the tumor onset. In *tgfα*/c-*myc* mice, this TGFα-induced cyclin D1 may have a twofold functionality (Figure 9). First, it may facilitate the early events (initiation and/or promotion) of the carcinogenic process, resulting in an earlier onset of tumors, when compared to single transgenic c-*myc* mice (Figure 9). Second, it may also contribute to the formation of a much faster-growing tumor phenotype, similar to what is discerned in the cyclin D1-positive foci within c-*myc* tumors. Moreover, in *tgfα*/c-*myc* mice the effect of TGFα on induction of cyclin D1 seems to override the suppression of cyclin D1 by c-Myc. This implies that TGFα and c-Myc may each regulate cyclin D1 as one step of their signaling pathways, and that cyclin D1 serves a pivotal role that links these two separate pathways. Cyclin D3 may not share this crucial property, as it is expressed not only in the tumor foci but also in the major tumor areas.

Expression of cyclin E in c-*myc* tumors is also reciprocal to that of c-Myc. This is surprising, as suppression of cyclin E by c-Myc has not been reported, and relevant literature suggests that c-Myc can activate expression of cyclin E *in vitro* (Amati *et al.*, 1998; Obaya *et al.*, 1999). Several studies have suggested that cyclin D1/cdk4 should be activated prior to the onset of cyclin E/cdk2 activity in order to ensure an orderly transition to S phase (Obaya *et al.*, 1999; Prall *et al.*, 1998). Thus, it is possible that the lack of a sufficient amount of cyclin D1 may hamper the expression of cyclin E. It is even possible that prevention of expression of cyclin E may facilitate the cell growth during the early stages of the carcinogenic process in c-*myc* animals, as it has been shown that stable overexpression of cyclin E, rendered by cDNA transfection, inhibits growth of mammary epithelial cells (Sgambato *et al.*, 1996). However, similar to what we have discussed for cyclin D1, once a tumor is formed, cyclin E overexpression may be required for its further progression to more aggressive phenotypes (Figure 9), as suggested by the observation that cyclin E-positive cells show a trend for more rapid proliferation and for penetration into their adjacent tumor areas. Additional support for this hypothesis is provided by the observation that the more-aggressive *tgfα*/c-*myc* tumors exhibit overexpression of cyclin E as well. The overexpression of cyclin E may result from a synergy between TGFα and c-Myc, because expression of cyclin E is not pronounced in the hyperplastic epithelium from either *tgfα* or c-*myc* animals. This hypothesis is consistent with the observation in human breast cancer, that overexpression of cyclin E is correlated with increased tumor grade (Nielsen *et al.*, 1996; Keyomarsi, *et al.*, 1994). Moreover, a well-known, but mechanistically-unclear phenomenon is that overexpression of the c-*myc* gene alone is insufficient for transformation of most types of cells either *in vitro* or *in vivo*; cooperation of c-*myc* with growth factors (like TGFα) or some oncogenes (such as

ras) greatly enhances its transforming efficacy (Valverius *et al.*, 1990; Schmidt, 1999; Facchini *et al.*, 1998; Amati *et al.*, 1998; Dang, 1999; Nass *et al.*, 1997). The reciprocal expression of c-Myc and cyclins D1 and E in c-*myc* tumors and the co-expression of these genes in *tgfα*/c-*myc* tumors raise the possibility that one role of these additional factors may be to rescue the expression of cyclin D1 and/or cyclin E. Overexpression of these cyclins may be beneficial for the transformation, but it may be hampered because of constantly high levels of c-Myc.

Our transgenic models reveal, for the first time, that cdk2β, but not cdk2α, occurs as the faster-migrating phosphorylated form (Gu *et al.*, 1992; Planas-Silva *et al.*, 1997) in a primary tumor tissue. Little is known about functions of cdk2β. Its expression has been shown to peak at S phase and decrease significantly at early G2 phase, in contrast to the expression of cdk2α, which usually shows little change through the entire cell cycle (Kotani *et al.*, 1995). Thus, it cannot be ruled out in c-*myc* and *tgfα*/c-*myc* tumors, that the predominant partner of cyclins A2 and E during S phase may be cdk2β.

Levels of the pRB protein are greatly decreased in the majority of *tgfα*/c-*myc* tumors, relative to c-*myc* tumors. This may occur at the mRNA level in some cases, as shown by RT–PCR analysis. For those *tgfα*/c-*myc* tumors in which the Rb mRNA and protein are detected, it is not yet clear if the expression is contributed by the tumor cells or by the proliferating stromal cells within the tumors. Regardless of the mechanism, loss of pRB protein may be one of the major reasons why mammary tumors in double transgenic mice develop at such early ages and grow at such a rapid rate, given the fact that pRB is a potent tumor suppressor and growth inhibitor. The loss of pRB in bi-transgenic tumors may be due to a synergy between c-Myc and TGFα, rather than an effect of TGFα alone, since *tgfα* mice do not develop tumors. This implies that like cyclin D1, pRB also links the c-Myc- and TGFα signaling pathways in control of cell cycle progression. However, cooperation between c-Myc and TGFα through cyclin D1 and pRB may be mechanistically different, since TGFα antagonizes the effect of c-Myc on cyclin D1 expression but appears to promote the effect of c-Myc on the attenuation of expression of pRB, as the pRB levels in some c-*myc* tumors are also low.

In conclusion, c-Myc may induce, directly or indirectly, expression of cyclin A2 and E2F1 as primary events to mediate the onset of mammary tumors in c-*myc* transgenic mice. In contrast, overexpression of cyclins D1 and E may occur as later events to facilitate progression of focal islands within the c-*myc* tumors to more aggressive phenotypes. Similarly, by using bi-transgenic mice, we concluded that TGFα induces cyclin D1 and facilitates the loss of pRB. These TGFα-mediated effects may have a threefold consequence in the mammary carcinogenesis of *tgfα*/c-*myc* bi-transgenic animals, relative to c-*myc* mice: a much earlier tumor onset, a higher tumor frequency, and the formation of a much more aggressive tumor phenotype. Thus, during mouse mammary carcinogenesis in bi-transgenic animals, TGFα and c-*myc* cooperate to control the cell cycle progression, in particular, at the levels of cyclin D1 and pRB.

Materials and methods

Tissue collections

MT-*tgfα*, MMTV-c-*myc*, and MT-*tgfα*/MMTV-c-*myc* single or double transgenic mice were generated, housed, and genotyped as described previously (Amundadottir *et al.*, 1995). Tissue materials used were from the previously reported studies (Amundadottir *et al.*, 1995), with additional animal experiments carried out in the same way. Briefly, virgin female MMTV-c-*myc* or MT-*tgfα* single transgenic mice were sacrificed at ages of 10–12 months, together with age-matched, non-transgenic mice of the parental strain (FVB). MT-*tgfα*/MMTV-c-*myc* double transgenic mice were sacrificed at ages of 3–6 months, together with five age-matched, non-transgenic animals as control. Mammary or tumor tissues harvested from the animals were either stored at −80°C or fixed with 10% buffered formalin and embedded in paraffin.

TUNEL assay

The terminal deoxynuycleotidyl transferase (TdT) mediated digoxigenin-dUTP nick end labeling (TUNEL) method was carried out using a kit from Trevigen Inc., Gaithersburg, MD, USA. Paraffin sections were labeled with TdT and biotin-labeled dNTP, and were incubated with peroxidase-conjugated Streptavidin, according to the manufacturer's instructions. The signal was visualized by exposure to diaminobenzidine and H_2O_2, followed by counter-staining with hematoxylin.

In situ *hybridization assay*

Paraffin sections were hybridized overnight at 60°C with riboprobes, transcribed from the antisense or sense strands of the cDNAs and labeled with digoxigenin-conjugated UTP, as described previously (Li *et al.*, 1999). The sections were then incubated with an antibody against digoxigenin, followed by incubation with a second antibody conjugated to alkaline phosphatase. The signal was visualized by color development with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All reagents were purchased from Boehringer Mannheim, Indianapolis, IN, USA. A 1.4 kb mouse c-*myc* cDNA and a 0.9 kb mouse *e2f1* cDNA (ATCC, Manassas, VA, USA) were used for labeling of the riboprobes. To control the signal specificity, two serial sections were mounted on the same slide for hybridization with antisense and sense probes, respectively. A serial selection was also pretreated with RNase A and then post-fixed with 4% formaldehyde to denature the RNase before hybridization with antisense probe.

Northern blot assay

Ten μg of total RNA per sample were loaded and electrofractionated in an agarose gel containing formaldehyde. Roughly equal loading of lanes and RNA integrity were confirmed by staining the gel with ethidium bromide. The separated RNA was transferred to nitrocellulose membranes and hybridized with an *e2f1* antisense riboprobe, synthesized from the same cDNA as used for *in situ* hybridization, and labeled with ^{32}P-ATP (Amersham Life Science, Inc., Arlington Heights, IL, USA). After washes with SSC buffers, the membrane was subjected to autoradiography.

RT–PCR analysis

Total RNA was reverse-transcribed and then amplified using the RT–PCR kit from GIBCO/BRL, Rockville, MD, USA. The conditions for the PCR amplification were as follows: 3-min hot start at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C. The two pairs of forward/

reverse PCR primers for the Rb gene were 209–229 bp/1110–985 pb and 1014–1041 pb/2857–2833 pb, which overlap to span most part of the mouse Rb mRNA (Bernards *et al.*, 1989). As an internal control, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified by PCR under the same conditions. The forward and reverse primers were 819–837 pb and 1228–1207 bp, respectively, of the mouse GAPDH cDNA sequence (Sabath *et al.*, 1990).

Western blot analysis

Methods for preparation of protein samples and for Western blotting were described previously (Liao *et al.*, 1998). Protein aliquots (20–80 μg per lane) were electro-fractionated on SDS–PAGE. Roughly equal loading was confirmed by staining the gel with Coomassie blue. One primary pRB antibody (14001A) was purchased from Pharmigen, San Diego, CA, USA and another (pRB245) was a generous gift from Dr W-H Lee (see Acknowledgements). The PCNA primary antibody (PC10) was purchased from Oncogene Research Product Inc., Cambridge, MA, USA. All other primary antibodies were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA): c-Myc (C19), E2F1 (C20 and KH95), cyclin A (C19), cyclin E (M-20), cyclin D1 (C20), cyclin D3 (C16), cdk2 (M20), cdk4 (C22), p16 (M156 and F12), p21 (M19 and F5), and p27 (C19 and N19). For all primary antibodies from Santa Cruz Biotech. Inc., where specific blocking peptides were available, in a parallel Western blot assay the antibody was incubated with fivefold excess (by weight) of the corresponding blocking peptide to neutralize the antibody before applied to the membrane. The pre-neutralized antibody sample did not give rise to the specific signals at correct molecular weights, demonstrating the specificity of the primary antibodies.

Immunohistochemical staining

A peroxidase-anti-peroxidase (PAP) method was used as described previously (Liao *et al.*, 1998). The primary antibodies were the same as used for Western blot analyses. For all primary antibodies purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA), where blocking peptides were available, in one staining with a serial section, the primary antibody was incubated with fivefold excess (by weight) of its blocking peptide for 2 h to neutralize the antibody before application to the section. The pre-neutralized primary antibody did not give rise to signal, demonstrating that the signal given by the primary antibody was specific.

Labeling index

Labeling indices for TUNEL and PCNA staining were determined for tumors from *tgfα*/c-*myc* mice and for specific tumor foci and their adjacent tumor areas from c-*myc* animals. Since cells in the G1 phase of the cell cycle manifest weak nuclear staining for PCNA, in strong contrast to the intense nuclear staining of cells in S phase (Eldrige *et al.*, 1993), only those cells displaying strong nuclear staining were counted. Four *tgfα*/c-*myc* tumors plus six foci and their adjacent tumor areas from different individual animals were counted. For each tumor or focus, three randomly selected areas, about 600 tumor cells per area, were counted. The percentage of labeled cells was calculated and presented as mean ± s.d. The χ^2 test of independence for an $r \times c$ contingency table was used for the statistical analysis.

Abbreviations

Cdk, cyclin-dependent kinase; ER, estrogen receptor; MMTV, mouse mammary tumor virus; MT, metallathionein; PCNA, proliferating cell nuclear antigen; pRB, retinoblastoma protein; TGFα, transforming growth factor α; TUNEL, terminal deoxynucleotidyl transferase nick end labeling.

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Appendix II

Endocrine-Related Cancer (2000) **7** 143–164

c-*Myc* in breast cancer

D J Liao[1,2] *and R B Dickson*[1,2,3]

[1]Vincent T Lombardi Cancer Center, Georgetown University Medical Center, Washington DC, USA
[2]Department of Oncology, Georgetown University Medical Center, Washington DC, USA
[3]Department of Cell Biology, Georgetown University Medical Center, Washington DC, USA

(Requests for offprints should be addressed to R B Dickson, Lombardi Cancer Center, Research Building W417, 3970 Reservoir NW, Washington DC 20007, USA; Email: dicksonR@gunet.georgetown.edu)

Introduction

Ever since Bishop and his co-workers discovered the c-*myc* gene in the late 1970s (Bishop 1982), voluminous literature has documented its central role in proliferation and malignant transformation of human and animal cells (Amati *et al.* 1998, Bouchard *et al.* 1998, Dang *et al.* 1999). Most, if not all, types of human malignancy have been reported to have amplification and/or overexpression of this gene, although the frequency of these alterations varies greatly among different reports (Nesbit *et al.* 1999). In 1992, researchers started to realize that aberrant expression of c-*myc* could cause apoptosis (Evan *et al.* 1992, Shi *et al.* 1992), although the phenomenon had actually been observed much earlier (Wurm *et al.* 1986). Studies in recent years have further shown that the c-*myc* gene regulates growth, both in the sense of cell size and in the context of tissue differentiation (Gandarillas & Watt 1997, Iritani & Eisenman 1999, Johnston *et al.* 1999, Schmidt 1999, Schuhmacher *et al.* 1999). Thus, it is now known that the c-*myc* gene participates in most aspects of cellular function, including replication, growth, metabolism, differentiation, and apoptosis (Packham & Cleveland 1995, Hoffman & Liebermann 1998, Dang 1999, Dang *et al.* 1999, Elend & Eilers 1999, Prendergast 1999). How the c-Myc protein may be specifically directed to perform one, but not the others, of these functions is still obscure, despite the fact that the relevant literature has been accumulating at a fast pace in the past two decades. This review focuses on the profound roles of c-Myc in breast cancer and in the actions of the hormones that are eitologically related to breast cancer.

The c-*myc* gene and c-Myc proteins

The c-*myc* gene is transcribed to three major transcripts that start from different initiating sites (Fig. 1), yielding three major proteins named c-Myc1, c-Myc2, and c-MycS (Henriksson & Luscher 1996, Facchini & Penn 1998, Xiao *et al.* 1998). c-Myc2 is an approximately 62-kDa protein that is the major form of the three c-Myc proteins and the one referred to as 'c-Myc' in most studies. c-Myc1 arises from an alternative initiation site at an in-frame, non-AUG codon, yielding a protein 2–4 kDa larger than c-Myc2. c-MycS arises from a leaky scanning mechanism, and initiates at two closely spaced downstream AUG codons, resulting in a protein lacking about 100 amino acids at the N-terminus of c-Myc2 (Claassen & Hann 1999). An unusual property of the c-*myc* gene that is often neglected by investigators is that the antisense strand of the gene also yields transcripts (Spicer & Sonenshein 1992). Therefore, one should exert caution when using antisense expression as the control for judging the level of c-*myc* mRNA.

The amino terminus of each full-length c-Myc protein (c-Myc1 and c-Myc2) harbors a transactivation domain (TAD), within which are two regions that are highly conserved among members of the Myc family; these regions are termed Myc homology boxes I and II (MBI and MBII) (Fig. 1). The carboxyl terminus of the c-Myc proteins contains a basic region and a helix-loop-helix/leucine zipper (HLH/LZ) domain. Through the HLH/LZ domain, a c-Myc protein heterodimerizes with another transcription factor, Max; the c-Myc/Max complex then binds to a specific DNA recognition sequence, the so-called E-box element that contains a central CAC(G/A)TG motif (Henriksson & Luscher 1996, Amati *et al.* 1998, Facchini & Penn 1998, Dang 1999). Genes containing this Myc E-box element in their regulatory regions may be c-Myc targets, and thus subjected to transactivation or transrepression by the c-Myc/Max complex (Cole & McMahon 1999). Within the TAD, the MBI has been shown to be required for the transactivation activities of c-Myc, whereas the MBII is needed for the trans-suppression activities (Cole & McMahon 1999, Sakamuro & Prendergast 1999). c-MycS lacks the MBI but still retains the MBII in its TAD; this may be the reason why c-MycS is deficient for transactivation but retains the activity of trans-suppression (Xiao *et al.* 1998, Sakamuro & Prendergast 1999). Thus, c-Myc1 and c-Myc2 can both activate and repress transcription of specific target genes, whereas c-MycS can only repress transcription and can thus function as a dominant-negative inhibitor of certain (but not all) activities of the full-length c-Myc proteins (Xiao *et al.* 1998, Sakamuro & Prendergast 1999).

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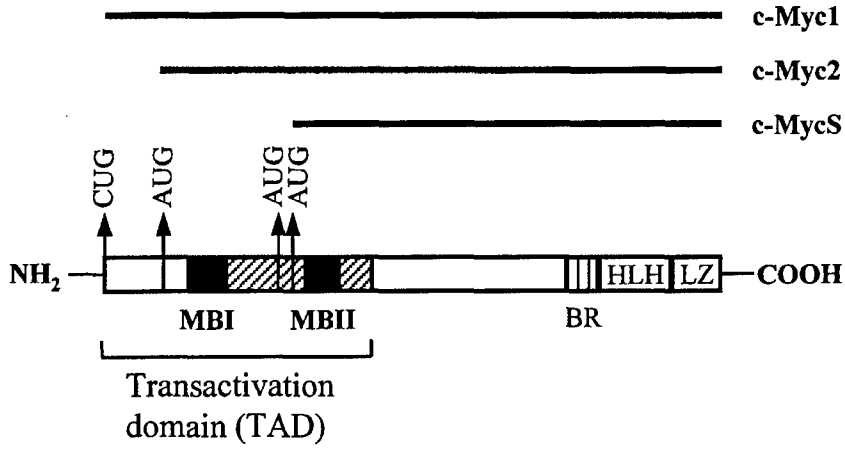

Figure 1 Schematic diagram of the Myc family proteins. Within the TAD at the N-terminus there are two *myc* homology boxes (MBI and MBII) which are conserved among Myc family proteins. At the C-terminus, the HLH/LZ domain links to the basic region (BR) of the c-Myc protein. The initiation sites of the three c-Myc proteins are indicated.

Under normal growth conditions, expression of c-Myc1 and c-Myc2 proteins is differentially regulated (Batsche & Cremisi 1999). In cell culture, c-Myc2 is synthesized in growing cells, while expression of c-Myc1 increases dramatically, to a level equal to or greater than that of c-Myc2 as the cells approach high density (Ryan & Birnie 1996). These observations lead to an hypothesis that in this context c-Myc1 may act as a growth inhibitor, expression of which may be triggered by contact-inhibition (Ryan & Birnie 1996). Regulation of c-MycS is much less known, relative to c-Myc1 and c-Myc2. It has been shown that its expression is increased to the levels comparable to those of c-Myc2 during rapid cell growth, and constitutively high levels of c-MycS have been found in some tumor cell lines as well (Spotts *et al.* 1997).

Role of c-Myc proteins in cell proliferation

In physiological situations, the central role of c-Myc may be its promotion of cell replication in response to extracellular signals, by driving quiescent cells into the cell cycle. This function was originally thought to be elicited mainly via activation of transcription of those c-Myc target genes that are positive regulators of the cell cycle (Amati *et al.* 1998), such as cyclins D1, D2, E and A, *cdk*4, *e2f*1, *e2f*2, *cdc25A* and *B*, etc. (Barrett *et al.* 1995, Amati *et al.* 1998, Ben-Yosef *et al.* 1998, Dang 1999, Dang *et al.* 1999). However, of these putative c-Myc target genes, only *cdk*4 (Hermeking *et al.* 2000), *e2f*2 (Sears *et al.* 1997), and cyclins D1 (Daksis *et al.* 1994) and D2 (Perez-Roger *et al.* 1999) seem to encompass a Myc E-box element in their regulatory regions. More confusingly, several *in vitro* studies show that c-Myc actually suppresses the transcription of the cyclin D1 gene (Jansen-Durr *et al.* 1993, Philipp *et al.* 1994). One explanation for this paradox may be that activation of most of these cell cycle components by c-Myc may be by indirect mechanisms. Other possibilities include the involvement of other c-Myc binding proteins, in addition to Max (Sakamuro & Prendergast 1999). Concurrent binding of these proteins may redirect the Myc/Max dimer to promoters with non-canonical E-box elements, or may stabilize the weak binding of the Myc/Max to other promoter sites (Claassen & Hann 1999, Sakamuro & Prendergast 1999). TRRAP (TRansformation/tRanscription domain Associated Protein) (McMahon *et al.* 1998), BIN1 (Box-dependent myc-INteracting protein–1 or Bridging INtegrator–1) (Elliott *et al.* 1999), and BRCA1 (Wang *et al.* 1998) are examples of such c-Myc-binding proteins. TRRAP seems to be required for Myc-mediated transformation (McMahon *et al.* 1998), whereas BIN1 bound to c-Myc inhibits Myc-mediated transformation (Elliott *et al.* 1999).

In principle, promotion of cell cycle progression by c-Myc can also be achieved by suppression of transcription of growth inhibitory genes (Alexandrow & Moses 1998). Examples of these genes include *gadd*45 (Marhin *et al.* 1997), cdk (cyclin-dependent kinase) inhibitors $p21^{cip1}$ (Mitchell & El-Deiry 1999, Coller *et al.* 2000), $p19^{ARF}$ (Dang 1999) and probably also $p27^{kip1}$ (Amati *et al.* 1998, Donjerkovic *et al.* 1999, Wu *et al.* 1999). There is evidence that under certain conditions the role of c-Myc in cell cycle progression may require only its activity of trans-suppression, not that of transactivation (Claassen & Hann 1999). For instance, c-MycS, which lacks the transactivation activity but retains the trans-suppression activity, can still promote proliferation of several types of

cells in culture (Xiao *et al.* 1998). Further, it has been suggested that the trans-suppression may actually be more important for cell proliferation than the transactivation (Claassen & Hann 1999), although this hypothesis still requires further experimental validation.

Roles of c-Myc in transformation

Transformation of a cell may not be a physiological function of c-Myc; rather, it may occur only when c-Myc is aberrantly expressed or genetically altered. Although transfection of c-Myc alone transforms Rat1 cells, as measured by an anchorage-independent growth assay, transformation of rat embryonic fibroblasts (REF) or certain primary cultured cells by c-Myc requires its co-transfection with another oncogene or growth factor gene, such as c-*ras*^{H} or transforming growth factor-α (*tgfα*) (Amati *et al.* 1998). In addition, temporary overexpression of c-Myc in Rat1 cells has been shown to induce genetic instability (Felsher & Bishop 1999). Since Rat1 cells are immortalized, it is generally believed that this property of Rat1 cells contributes to their unusual susceptibility to c-Myc-induced transformation and genetic alteration. Like c-Myc2, c-MycS alone is able to transform Rat1 cells. Unlike c-Myc2, however, c-MycS cannot cooperate with c-*ras*^{H} to transform REF (Xiao *et al.* 1998). Our laboratory has also found that c-MycS fails to induce DNA damage-induced check-point abrogation (Sheen & Dickson 2000). One explanation for these results is that transformation of REF by c-*myc*/c-*ras* cotransfection requires transactivation of certain proliferation-related genes to immortalize the cells as the priming step of transformation. c-Myc2 can induce proliferation of both REF and Rat1, whereas c-MycS can only induce proliferation of Rat1 (Xiao *et al.* 1998). These data further imply that some genes required for the replication of mortal cells may not be needed for the replication of immortalized cells, and that activation of these genes requires transactivation by c-Myc2. Perhaps the ability of c-Myc2 to immortalize a cell is required to make REF cells susceptible to transformation by c-*ras*^{H}, as c-*ras*^{H} has been shown to induce senescence (Serrano *et al.* 1997). Since c-*ras*^{H} is a signaling molecule engaged by many growth factors, one may further surmise that the requirement of co-transfection with growth factor genes or other oncogenes may also be due to the requirement of activation of proliferation-related genes for immortalizing the cells. It remains to be determined whether the c-Myc-induced genetic instability also requires its transactivation ability and contributes to its transformation activities.

The role of c-Myc in transformation may be directly related to its regulation of expression of the human telomerase transcriptase gene (*hTERT*) (Greenberg *et al.* 1999), since telomerase functions to immortalize cells. Analysis of the 5′-flanking sequence of *hTERT* further reveals that transcription of this gene is dependent on a proximal 181 bp region of the promoter, which is essential for its transactivation in immortalized and cancer cells (Oh *et al.* 1999). This promoter region contains c-Myc E-boxes and GC-boxes (the consensus binding sequence for Sp1), and thus presumably is responsible for the observed cooperation between c-Myc and Sp1 in transcriptional activation of the *hTERT* gene (Oh *et al.* 1999, Kyo *et al.* 2000). In addition, estrogen has also been shown to activate *hTERT*, in part via its activation of the expression of c-Myc (Kyo *et al.* 1999). On the other hand, *hTERT* is also transcriptionally repressed by Mad (Gunes *et al.* 2000, Oh *et al.* 2000), a protein that can compete with c-Myc in binding to Max, via directly binding to the *hTERT* promoter. Since the Max/Mad complex acts in an antagonistic manner to c-Myc/Max-induced transactivation, elevation in Mad is anticipated to suppress *hTERT* expression also indirectly by decreasing both the abundance of the c-Myc/Max complex and the transactivation activity of c-Myc/Max.

Roles of c-Myc in apoptosis and their connection to carcinogenesis

Two sets of conflicting phenomena have frequently been reported in the literature pertaining to the role of c-Myc in apoptosis. (1) Constant overexpression of c-Myc by the approaches of transfection, viral-infection or transgenic animals may induce apoptosis (Packham & Cleveland 1995, Alarcon *et al.* 1996, Hagiyama *et al.* 1999, Prendergast 1999), usually following or associated with cell proliferation (Hoffman & Liebermann 1998), whereas cells transfected with c-*myc* antisense oligodeoxynucleotides to decrease the c-*myc* levels become resistant to apoptotic stimulus (Lee *et al.* 1997). (2) A decrease in c-Myc levels by techniques such as an antisense approach may also cause apoptosis of certain tumor tissues or tumor cells (Balaji *et al.* 1997, Citro *et al.* 1998, Putney *et al.* 1999, Wu *et al.* 1999), or may increase the sensitivity of the cells to apoptotic stimuli (Kang *et al.* 1996, Rupnow *et al.* 1998, Loffler *et al.* 1999). In addition, constant overexpression of c-*myc* has also been shown to reduce sensitivity to UV-induced apopotosis (Waikel *et al.* 1999). These conflicting observations suggest that c-Myc is capable of both inducing and suppressing apoptosis. To induce a tumor, c-Myc may need not only to promote cell proliferation but also simultaneously to inhibit its tendency for cell death, so as to increase the cell number to form a tumor mass (Cory *et al.* 1999, Lowe & Lin 2000). Therefore, the role of c-Myc in inhibiting apoptosis is easily connected to its tumorigenicity. Whether and how c-Myc-induced apoptosis also contributes to carcinogenesis is much less clear, although, in general, apoptosis is suggested to accelerate cell turnover and thus facilitate the progression of cells to more and more malignant phenotypes during the carcinogenic process (Vakkala *et al.* 1999). A puzzle is that, although c-Myc is frequently overexpressed in various tumor

tissues in human and in animals under spontaneous or experimental conditions, little, if any, evidence has shown that the apoptosis appearing in these tumors is related to the increased levels of c-Myc.

The foundation of the concept of 'c-Myc-induction of apoptosis' is built mainly on the systems where c-Myc expression is induced in a constant manner by approaches of transfection, viral-infection, or a specific promoter-driven c-*myc* transgene, while the expression level of the endogenous c-Myc is down-regulated (Prendergast 1999, Packham & Cleveland 1995). These artificial systems are different from the physiological situation in which c-Myc expression arises under its own promoter, specifically during G0/G1 transit of the cell cycle. Perhaps a drop in the levels of c-Myc later in the cell cycle is required for the rise of other proliferation-related genes, such as G1 cyclins. Thus, a constantly high level of c-Myc may disrupt the cyclic pattern of expression of these genes; the cell may then be signaled to die. Currently, it is still technically impossible to test this hypothesis, since it is impossible to induce c-Myc transiently and specifically at the G0/G1 transit. Moreover, in the *in vivo* situation, a cell that has already proliferated may remain untransformed, or it may be transformed but still retain certain critical differentiated features such as contact-inhibition. Such a cell may be terminated through an apoptotic pathway in order to maintain a particular physiological condition, such as the normal size of the organ. This may be one of the reasons why c-Myc-overexpressing cells, which usually have already undergone proliferation, may commit apoptosis.

How c-Myc induces apoptosis is still unclear, despite the fact that many apoptotic pathways, such as those that are p53-dependent and -independent, have been suggested by different experimental systems (Packham & Cleveland 1995, Hagiyama *et al.* 1999, Prendergast 1999, Soengas *et al.* 1999). The transactivation domain of the c-Myc protein has been shown recently to modulate the apoptosis directly (Chang *et al.* 2000). Another study shows that C-MycS retains the ability to induce apoptosis of several types of cells, and the MBII of c-MycS is needed for this function (Xiao *et al.* 1998), indicating that in certain situations c-Myc-induced apoptosis may require trans-suppression, but not transactivation, of c-Myc proteins.

The c-*myc* gene in human breast cancer

As shown in our recent meta-analysis (Deming *et al.* 2000) and in Table 1 that lists the most references found by search in Medline, a range of 1 to 94%, 15.5% on average, of breast cancer biopsies bear c-*myc* gene amplification of threefold or greater. The great variation in the frequencies may be attributed to the low sensitivity of some methods used (Soini *et al.* 1994), the tumor grades studied, and the small number of cases in many of the studies. Early diagnosis of many cases may be another reason, as the gene may be amplified during both early and late stages of cancer progression. The reported frequencies of overexpression of c-*myc* are also greatly variable. A recent report reveals that only about 22% of the tumor cases show increased c-*myc* mRNA expression, and the overexpression was rarely due to the gene amplification (Bieche *et al.* 1999). However, several other studies (Table 1) show much higher percentages of breast cancer cases with mRNA overexpression (Guerin *et al.* 1988, Mariani-Costantini *et al.* 1988, Tervahauta *et al.* 1992, Escot *et al.* 1993, Nagai *et al.* 1994, Le *et al.* 1999). Some of these studies suggest that the overexpressed mRNA might be related to gene amplification (Mariani-Costantini *et al.* 1988). Since most of these mRNA studies were carried out using Northern blot, dot blot, or PCR-based techniques with tissue lysates, but not using *in situ* hybridization, the increase in the expression may actually be assessed with great bias. This is because normal breast tissue is dominated by fat tissue; it differs greatly from tumor tissue in its epithelial cellularity, and thus is not a rigorously normal counterpart for comparisons involving mRNA extraction.

As listed in Table 1, many studies utilizing immunohistochemistry show that about 50–100% of breast cancer cases have increased levels of c-Myc proteins (Agnantis *et al.* 1992, Pavelic *et al.* 1992*a*,*b*, Saccani *et al.* 1992, Hehir *et al.* 1993, Spaventi *et al.* 1994, Pietilainen *et al.* 1995). In one of those reports, 95% of the cases show positive staining of c-Myc in the cytoplasm, and only 12% of the cases reveal either nuclear or both nuclear and cytoplasmic staining (Pietilainen *et al.* 1995). Other investigations also report a predominantly cytoplasmic localization of c-Myc proteins, although nuclear localization is also observed (Mizukami *et al.* 1991). The meaning of the cytoplasmic localization is currently unknown. Regardless of the cellular location of c-Myc, it seems that there is a higher percentage of breast cancer cases showing aberrant c-Myc protein levels than the percentage with the gene amplification. This implies that, in many cases, altered expression or altered stability of the mRNA or protein may be the mechanism for the increased levels of c-Myc, consistent with the initial report that c-Myc overexpression precedes its gene amplification and plays a role in amplification of multiple other genes and in other chromosomal instability events (Mai 1994, Mai *et al.* 1996).

In breast cancer, amplification of c-*myc* may correlate positively or negatively with alterations in other genes (Courjal *et al.* 1997, Cuny *et al.* 2000). The amplification of the chromosomal region that contains the c-*myc* gene has also been reported to contain, apparently through translocation events, the p40 subunit of eukaryotic translation initiation factor 3 (*eIF3*) and the *Her2* gene (Nupponen *et al.* 1999), although the frequency of co-localization of these two genes in a common amplicon has not been established (Deming *et al.* 2000). *Her2* (also termed *erbb2*) is a gene in

Table 1 Summary of the amplification, RNA or protein expression of c-*myc* in human breast cancer

Reference	Methods	Main c-*myc*-related conclusions
Escot *et al.* (1986)	SN,NB	Not associated with ER or PR status
Whittaker *et al.* (1986)	Dot-B	Expressed in both benign lesions and cancer
Cline *et al.* (1987)	SB	More common in recurrent tumors
Spandidos *et al.* (1987)	IHC	Expressed in both benign lesions and cancer
Varley *et al.* (1987*a*)	SB	Correlated with poor prognosis
Varley *et al.* (1987*b*)	SB	Identified a rearrangement with deletion
Biunno *et al.* (1988)	SB,NB,Slot-B	No clinicopathologic parameters mentioned
Bonilla *et al.* (1988)	SB,NB	Not correlated with clinicopathologic parameters
Guerin *et al.* (1988)	SB,NB	Correlated with poor prognosis; co-expressed with *Her2*
Mariani-Costantini *et al.* (1988)	ISH	RNA overexpression related to the gene amplification
Morse *et al.* (1988)	SB	Gene rearrangement and mutation
Adnane *et al.* (1989)	SB	Correlated with high grade and PR^-
Garcia *et al.* (1989)	SB	Associated with the inflammatory type of the cancer
Gutman *et al.* (1989)	SB	Not related with tumor stage or prognosis
Locker *et al.* (1989)	IHC	Not related to any clinicopathologic parameters
Machotka *et al.* (1989)	SB	More frequently in patients with node metastases
Spandidos *et al.* (1989*a*)	ELISA	Not correlated with survival
Spandidos *et al.* (1989*b*)	IHC	Not correlated with node metastasis
Tauchi *et al.* (1989)	IHC	Not correlated with ER, tumor histology or sizes
Tavassoli *et al.* (1989)	SB,Slot-B	Correlated with tumour grade, but not with metastasis
Tsuda *et al.* (1989)	Slot-B	Correlated with poor prognosis
Walker *et al.* (1989)	ISH,IHC	No correlation among amplification, RNA and protein
Guerin *et al.* (1990)	NB	Associated with poor prognosis
Meyers *et al.* (1990)	SB	Only 1% frequency of amplification
Tang *et al.* (1990)	SB	Associated with lymphocyte infiltration of the tumors
Mizukami *et al.* (1991)	IHC	Correlated with ER^+, but not with clinical parameters
Escot *et al.* (1991)	ISH	Methodology on RNA quantitation
Fukutomi *et al.* (1991)	IHC	Related to cell surface sugar chains
Le Roy *et al.* (1991)	ISH	The RNA expression reduced by tamoxifen
Paterson *et al.* (1991)	Slot-B	Existence of co-amplification with *Her2*
Pavelic *et al.* (1991)	IHC	Mainly nuclear location; related to positive nodes
Tauchi *et al.* (1991)	IHC	Do not regulate HSP70 expression
Tsuda *et al.* (1991)	Slot-B	Amplification in both primary and metastatic tumors
Agnantis *et al.* (1992)	IHC	Elevated in benign lesions, as a pre-cancer marker
Berns *et al.* (1992*a*)	SB	Associated with high copies of IGF1R amplication
Berns *et al.* (1992*b*)	SB	Correlated with tumor size & node-positivity, not ER
Berns *et al.* (1992*c*)	SB	Inversely correlated with *neu* amplification and PR
Berns *et al.* (1992*d*)	SB	Correlated with shorter survival
Borg *et al.* (1992)	SB,Slot-B	Related to early recurrence and death, not to ER
Pavelic *et al.* (1992*a*)	IHC	More frequent in invasive tumors
Pavelic *et al.* (1992*b*)	IHC	Mainly nuclear location; related to positive nodes
Roux-Dosseto *et al.* (1992)	SB	Correlated with early recurrence
Saccani *et al.* (1992)	IHC	Not related to nodal status, ER or PR
Tervahauta *et al.* (1992)	PCR,ISH,IHC	No clinocopathologic parameters mentioned
Bootsma *et al.* (1993)	SB	Not associated with somatostatin receptor expression
Escot *et al.* (1993)	ISH	Higher expression in post-ovulatory phase
Gaffey *et al.* (1993)	SB	Co-amplified with *Her2*
Hehir *et al.* (1993)	IHC	Also overexpressed in benign lesions
Henry *et al.* (1993)	SB	Associated with poor tumor differentiation, not prognosis
Kreipe *et al.* (1993)	SB	Related to proliferation
Nagayama & Watatani (1993)	SB	Related to node metastases & tumor sizes
Ottestad *et al.* (1993)	SB	Only 1.1% amplification frequency
Pertschuk *et al.* (1993)	Slot-B,IHC	Correlated with recurrence
Yamashita *et al.* (1993)	SB	Related to tumor size, but not clinical parameters
Watson *et al.* (1993)	SB,PCR	Related to early progression
Bieche *et al.* (1994)	SB	Associated with loss of heterozygosity on 1p32
Bolufer *et al.* (1994)	SB	Associated with neu amplication and ER^-
Breuer *et al.* (1994)	IHC,WB	c-Myc protein elevated in both tumors and serum
Champeme *et al.* (1994*a*)	SB	Not related to metastasis-free survival

Table 1 Continued

Reference	Methods	Main c-*myc*-related conclusions
Champeme *et al.* (1994*b*)	SB	Not correlated with survival
Haranda *et al.* (1994)	SB	Not related to clinicopathologic parameters
Nagai *et al.* (1994)	NB	Not correlated with ER RNA levels
Spaventi *et al.* (1994)	IHC	Not related to clinicopathologic parameters
Pechoux *et al.* (1994)	SB,ISHS,IHC	Also overexpressed in benign lesions
Soini *et al.* (1994)	SB,ISH	No clinicopathologic parameters mentioned
Berns *et al.* (1995*a*)	SB	Related to relapse
Berns *et al.* (1995*b*)	SB	Inversely related to the *Rb* gene alteration
Bland *et al.* (1995)	IHC	Not related to recurrence
Brotherick *et al.* (1995)	Cytometry	Associated with *c-erbb3* expression
Contegiacomo *et al.* (1995)	SB	Not related to clinopathologic parameters
Correnti *et al.* (1995)	Slot-B	No clinicopathologic parameters mentioned
Ito *et al.* (1995)	SB	Not associated with ER or PR status
Janocko *et al.* (1995)	Dot-B	No clinicopathologic parameters mentioned
Lizard-Nacol *et al.* (1995)	SB	Amplification only in cancer, not in benign lesions
Lonn *et al.* (1995)	PCR	Correlated with survival
Pietilainen *et al.* (1995)	IHC	Related to better survival; mainly in cytoplasm
Ried *et al.* (1995)	CGH	Amplification found; no clinicopathology mentioned
Berns *et al.* (1996)	SSCP	Related to poor prognosis
Courjal & Theillet (1997)	CGH	CGH more sensitive than SB
Courjal *et al.* (1997)	SB	Correlated with ER^-
Persons *et al.* (1997)	FISH	Related to S phase and ER^-
Visscher *et al.* (1997)	FISH	No clinicopathologic parameters mentioned
Kononen *et al.* (1998)	Arrays	Amplification detected in tissue microarrays
Mimori *et al.* (1998)	RT-PCR	Correlated with ODC expression
Stanta *et al.* (1998)	RT-PCR	No clinicopathologic parameters mentioned
Bieche *et al.* (1999)	RT-PCR	Correlated with tumor size but inversely with survival
Le *et al.* (1999)	NB	Related to positive nodes
Nupponen *et al.* (1999)	SSH	Associated with *eIF3*-amplification
Schraml *et al.* (1999)	FISH	Amplification detected in tissue-microarray
Scorilas *et al.* (1999)	SB,NB	Related to survival and local recurrence
Sierra *et al.* (1999)	IHC	Related to metastasis when Bcl-2 also increased
Vos *et al.* (1999)	SB,CGH	No amplification in DCIS
Cuny *et al.* (2000)	SB	Correlated with ER^- and PR^-, but not with prognosis
Han *et al.* (2000)	IHC	Inversely correlated with Mad1 expression
Jonsson *et al.* (2000)	WB	Not related to Beta-catenin
Rao *et al.* (2000)	PCR	Up to 94% biopsies with amplification
Sierra *et al.* (2000)	IHC	Not related to clinicopathologic parameters

CGH, comparative genomic hybridization; Dot-B, dot blot; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry; ISH, *in situ* hybridization; NB, northern blot; RT-PCR, reverse transcription and PCR; SB, southern blot; Slot-B, slot blot; SSCP, single-strand conformation polymorphism; SSH, suppression substractive hybridization; WB; western blot; IGF1R, insulin-like growth factor-1; DCIS, ductal carcinoma *in situ*.

the epidermal growth factor receptor (EGFR) family and is amplified in about 20–30% of human breast cancer biopsies (Gaffey *et al.* 1993, Bolufer *et al.* 1994, Cuny *et al.* 2000). Moreover, some investigations have shown that amplification of *Her*2 and c-*myc* genes is positively correlated or simultaneously occurs in certain breast cancer biopsies (Guerin *et al.* 1988, Gaffey *et al.* 1993, Bolufer *et al.* 1994). However, inverse correlation between amplification of *Her*2 gene and c-*myc* has also been reported in a few other studies (Berns *et al.* 1992*c*, Sears *et al.* 1997).

Several studies show that amplification or over-expression of c-*myc* occurs more frequently in the cases that are negative for estrogen receptor (ER-) (Persons *et al.* 1997, Bolufer *et al.* 1994) and/or progesterone receptor (PR-) (Adnane *et al.* 1989, Berns *et al.* 1992*c*), although other investigations do not find such inverse correlation or even show the opposite correlation (Table 1). In our recent meta-analysis, only the correlation of c-*myc* amplification with PR negativity was of statistical significance (Deming *et al.* 2000). Amplification of the cyclin D1 gene (*ccnd1*) is also seen frequently in human breast cancer, which occurs preferentially in the cases without c-*myc* amplification (Barnes & Gillett 1998). Although this reciprocal amplification seems to be consistent with the *in vitro* observation that c-Myc represses the transcription of cyclin D1, direct evidence is still lacking for a reciprocal

relationship between the expression of c-Myc and that of cyclin D1 in any human cancer tissue.

In cultured breast cancer cells, c-Myc is able to mimic estrogen to induce cyclin E/cdk2 activity by maintaining the $p27^{kip1}$ in the cyclin D1/cdk4 complex (earlier in the cell cycle), so as to keep the cyclin E/2 complex free from $p27^{kip1}$-binding (Prall *et al.* 1998). Pathological data also show that levels of $p27^{kip1}$ and cyclin D1 are associated with each other in breast cancer (Gillett *et al.* 1999, Leong *et al.* 2000). Other roles of c-Myc to activate cyclin E/cdk2 activity include its direct induction of expression of cyclin E, its possible induction of an as yet unidentified factor to sequester the $p27^{kip1}$ from binding to the cyclin E/cdk2, its promotion of the ubiquitin-degradation of $p27^{kip1}$, and its possible repression of the expression of $p27^{kip1}$ (Amati *et al.* 1998, Donjerkovic *et al.* 1999, Obaya *et al.* 1999, Wu *et al.* 1999). Based on these *in vitro* data, a higher level of c-Myc would be expected to be associated with lower levels of $p27^{kip1}$. Since several reports have shown that a lower $p27^{kip1}$ level predicts a poorer prognosis of breast cancer (Cariou *et al.* 1998, Gillett *et al.* 1999), this is surmised to be related partly to the elevated levels of c-Myc. Currently, however, no studies have been reported to prove or disprove this speculation on the relationship between c-Myc and $p27^{kip1}$ and on their combined effect on the outcome of breast cancer. However, it has been shown in patients with cervical cancer that those with a low $p27^{kip1}$ level and a high c-Myc level survive longer than those with low levels of both $p27^{kip1}$ and c-Myc (Dellas *et al.* 1998).

Breast cancer-associated gene 1 (*BRCA*1) is a putative tumor suppressor gene, loss or inactivation of which especially increases the risk of breast and ovarian cancer (Deng & Scott 2000). BRCA1 can physically bind to the c-Myc protein and repress c-Myc-mediated transcription (Wang *et al.* 1998). Moreover, BRCA1 can reverse the phenotype of REF transformed by activation of c-Myc and c-Ras (Wang *et al.* 1998). These data indicate that the mechanism for BRCA1 to function as a tumor suppressor may be related, in part, to its binding to c-Myc and its repression of the transcriptional activity of c-Myc. Loss of *BRCA*1 is therefore expected to result in relatively increased c-Myc activity and transforming potential. It would be interesting to test this speculation in familial human breast cancer of *BRCA*1 carriers.

PTEN (phosphate and tensin homolog deleted on chromosome ten) is a tumor suppressor gene that is inactivated in a number of tumor types, including breast cancer (Ali *et al.* 1999, Di Cristofano & Pandolfi 2000). In a *myc*-CAT (chloramphenicol acetyl transferase) reporter gene system, ectopic expression of PTEN has been shown to repress transcription of c-*myc* in MCF–7 and MDA-MB 486 breast cancer cells (Ghosh *et al.* 1999), indicating that among its activities, PTEN may be a transcription factor and c-*myc* may be its target gene. The repression of c-*myc* by PTEN in these cells is coupled with increased apoptosis and with growth inhibition of the tumor developed from these cells in nude mice (Ghosh *et al.* 1999). It is thus conceivable that the tumor suppressive role of PTEN may be exerted, in part, by down-regulation of c-*myc*. It would be interesting to test this speculation in familial breast cancers of *PTEN* carriers (Cowden's syndrome).

The c-*myc* gene and mammary gland carcinogenesis in transgenic mouse models

Transgenic mice have been generated to target the c-*myc* gene to the mammary glands by placing the transgene under the control of the long terminal repeat of the mouse mammary tumor virus (MMTV) or the whey acidic protein (WAP) promoters (reviewed in Amundadottir *et al.* 1996*a*, Nass & Dickson 1997). In MMTV-c-*myc* transgenic mice, the transgene is expressed at high levels, specifically in the mammary and salivary glands of females (Stewart *et al.* 1984). Spontaneous carcinomas develop in mammary glands at a frequency of roughly 50% at about one year of age in the virgin females; distant metastasis is rare (Amundadottir *et al.* 1995, 1996*a,b*, Rose-Hellekant & Sandgren 2000). Males do not develop the tumors. Multiple pregnancies significantly increase the incidence and shorten the tumor latency (Stewart *et al.* 1984, Amundadottir *et al.* 1995, 1996*a,b*, Nass & Dickson 1997), indicating that certain physiological growth stimuli to the mammary gland, such as estrogen or progesterone, may serve as promoters of carcinogenesis. Female WAP-c-*myc* mice, on the other hand, must undergo pregnancy to develop mammary carcinomas (Sandgren *et al.* 1995). The incidence and tumor latency are thus likely to depend on the rounds of pregnancies. In the WAP-c-*myc* model, pregnancy is required for activation of the promoter, but it complicates the model as well, since pregnancy may also provide additional promotion of carcinogenesis, as seen in MMTV-c-*myc* mice.

Since in these c-*myc* transgenic models the latency periods for tumor onset are long and the tumor incidences are relatively low, the c-*myc* gene itself may not be sufficient for the induction of carcinogenesis; other endogenous promoting factors such as female sex hormones may still be required to complete the carcinogenic process. This hypothesis is in line with the *in vitro* experiments, showing that transfection of cells with c-*myc* alone fails to transform cells, and that co-transfection with another oncogene or growth factor gene is required. Consistent with the co-transfection experiments, double transgenic mice carrying c-*myc* and another gene, such as c-ras^{H}, *tgfα*, *bcl*–2, or c-*neu* develop mammary carcinomas at much higher frequencies and at earlier ages (reviewed in Amundadottir *et al.* 1996*a*, Nass & Dickson 1997).

Virgin female mice transgenic with MMTV-c-*neu*, the mouse counterpart of *Her*2, develop mammary carcinomas as well, with a 50% incidence at about 7 months, slightly earlier than that in MMTV-c-*myc* mice (Muller *et al.* 1988, Cardiff *et al.* 1991). However, about 90% of the MMTV-c-*neu*/MMTV-c-*myc* double transgenic mice, generated by mating these two strains, develop cancer at 4.6 months, a much shorter latency than the single transgene carriers (Muller *et al.* 1988, Cardiff *et al.* 1991). In human breast cancer, co-amplification of *Her*2 and c-*myc* has also been reported to be associated with a reduced survival in some studies (Gaffey *et al.* 1993, Bolufer *et al.* 1994, Cuny *et al.* 2000). Interestingly, *in vitro* c-*myc* has been shown to repress transcription of c-*neu* and reverse c-*neu*-induced transformed morphology of cultured NIH 3T3 cells (Suen & Hung 1991). Probably when a situation occurs to allow these two oncogenes to cooperate rather than to antagonize each other, it results in a more aggressive tumor type. Such a situation occurs in the double transgenic animals where both transgenes are driven by a transgenic MMVT promoter, and may also occur when both genes are co-amplified and/or translocated during development of human breast cancer.

MMTV-v-*ras*^{H} transgenic mice develop mammary adenocarcinomas at 50% frequency at an average latency period of 10 months for males and 5.6 months for virgin females (Sinn *et al.* 1987). The tumors from v-*ras* or c-*neu* transgenic mice bear some genetic alterations that are absent in the tumors from c-*myc* transgenic mice (Morrison & Leder 1994), indicating that the carcinogenesis initiated by these oncogenes may undergo different pathways. MMTV-v-*ras*^{H}/MMTV-c-*myc* double transgenic mice develop mammary tumors at 50% incidence after an average latency of 46 and 100 days in females and males respectively (Sinn *et al.* 1987), again showing a synergistic effect of both oncogenes. One disadvantage of the v-*ras*^{H}/c-*myc* and c-*neu*/c-*myc* dual transgenic models for the study of multistaged carcinogenesis is that, of the two oncogenes, it is difficult to tell which one is the major carcinogenic factor and which one is the synergist, since female carriers of either transgene develop the tumors.

Dual carriers of metallothionein–1 promoter-(MT)-*tgf*α and MMTV-c-*myc* also develop mammary gland carcinomas at virtually 100% frequency in both male and female mice (Amundadottir *et al.* 1995, 1996*a*,*b*). The latency for the appearance of frank tumors in both sexes is similar, about 66 days in our studies. Unlike MMTV-v-*ras*^{H} and MMTV-c-*neu* single transgenic mice, virgin female MT-*tgf*α mice develop only moderate hyperplasia in their mammary epithelium (Sandgren *et al.* 1990, Amundadottir *et al.* 1995), although a low frequency of mammary tumors may occur after the mice undergo multiple pregnancies (Sandgren *et al.* 1995, Humphreys & Hennighausen 2000). This characteristic defines a synergistic role for the transforming growth factor-α (TGFα) in this double transgenic virgin model. The *tgf*α/c-*myc* tumors also grow faster than the c-*myc* tumors. In addition, the equal incidence and latency in both sexes of animals suggest that the strong synergism between the two transgenes may take place at exceedingly early ages, when sex differentiation in the endocrine environment has not been well-established nor effective. Moreover, before a macroscopic tumor appears, all cells in the hyperplastic mammary glands from MT-*tgf*α/MMTV-c-*myc* mice have already displayed displastic morphology. These properties suggest a unique feature that, although neither *tgf*α nor c-*myc* alone is a sufficient carcinogenic factor, their synergism is potently carcinogenic and can transform mammary epithelial cells as early as during the early stages of development.

Parous female mice carrying dual WAP-*bcl*–2/MMTV-c-*myc* transgenes develop mammary cancer at an average latency period of 3.3 months, slightly, but significantly shorter than the latency (4.3 months) of tumors in parous female MMTV-c-*myc* carriers, whereas parous female WAP-*bcl*–2 mice do not develop cancers (Jager *et al.* 1997). There is evidence suggesting that *bcl*–2 and c-*myc* may also be synergistic in human breast cancer (Sierra *et al.* 1999). Interestingly, this synergism in mammary carcinogenesis is opposite to the antagonism in liver carcinogenesis observed in the *bcl*–2/c-*myc* double transgenic mice (de La *et al.* 1999). The reason behind this organ difference is unclear.

Loss of one p53 allele seems to have little effect on c-Myc carcinogenicity, since female MMTV-c-*myc*/*p53*+/– dual carriers develop mammary tumors at a frequency and a latency period similar to that seen in their MMTV-c-*myc* counterparts (McCormack *et al.* 1998). Study of the mammary carcinogenesis in MMTV-c-*myc*/*p53*–/– mice is unfortunately impossible, because of very early development of lymphomas (Elson *et al.* 1995, McCormack *et al.* 1998). However, these mice manifest dramatic hyperplasia in the mammary gland at an earlier stage than the MMTV-c-*myc* mice (McCormack *et al.* 1998), suggesting the possibility that lack of both *p53* alleles may still have certain promoting effects on c-Myc-induced mammary carcinogenesis, although the synergy would be much weaker compared with the synergistic effects in lymphomagenesis (Blyth *et al.* 1995, Elson *et al.* 1995).

Distinctive morphology of mammary tumors in transgenic animals

In a series of analyses, Cardiff and coworkers (Cardiff *et al.* 1991, 2000, Cardiff & Munn 1995, Cardiff & Wellings 1999) and Halter *et al.* (1992) noticed that the mammary tumors developed in various different transgenic mouse models manifest distinctive morphology. While more than 95% of the mammary tumors occurring spontaneously in ordinary laboratory mice can be categorized using the Dunn classification, only 9% of the tumors from various transgenic

mice could be placed into standard categories by this classification (Cardiff *et al. 2000). Over 90% of the c-myc* transgene-induced tumors are glandular large cell carcinomas, and the rest are adenocarcinomas (Cardiff *et al.* 1991, Cardiff & Munn 1995). Mammary tumors developed from parous MT-*tgfα* and parous MMTV-*tgfα* mice are all adenocarcinomas. While we also observed similar results, we noticed that c-*myc*/*tgfα* dual transgenic tumors were mainly acinar carcinomas, according to the classification of Cardiff *et al.* (2000), which was quite different from the c-*myc* or *tgfα* tumors. These morphological features suggest that tumor phenotype may reflect genotype, as proposed by Cardiff and coworkers. It is an intriguing question why the *tgfα*/c-*myc* double transgenic mice have a tumor phenotype different from that in either *tgfα*- or c-*myc* only mice. Does it mean that each of the *tgfα*, c-*myc*, and *tgfα*/c-*myc* genotypes selects its own favorable target cells in the mammary glands as the tumor progenitor? Clarification of this question will improve our understanding of how *tgfα* and c-*myc* cooperate in the *in vivo* situation.

The mammary tumors arising in virgin female c-*myc* transgenic mice are characterized by the large number of apoptotic cells (Amundadottir *et al.* 1996*b*, Hundley *et al.* 1997, Bearss *et al.* 2000, Liao *et al.* 2000). About 15% of the tumor cells are apoptotic as identified by TUNEL staining, in strong contrast to the 1–2% in *tgfα*/c-*myc* double transgenic tumors (Liao *et al.* 2000). In c-*myc* tumors, the apoptotic cells are organized in clusters, which in sections stained by the TUNEL method are manifested as many small stained islands among non-apoptotic cells (see Fig. 2 in Liao *et al.* 2000). This morphology differs greatly from the random spreading of the proliferating tumor cells labeled by proliferating cell nuclear antigen (PCNA) in the same tumor. This difference in the histological organization of apoptotic cells and proliferating cells may be c-Myc-related, rather than mammary gland-specific, since similar 'clusters' of apoptotic cells are also discerned in the renal ducts that express a c-*myc* transgene (Trudel *et al.* 1997). One of the logical explanations may be that a paracrine or juxtacrine mechanism is involved in the c-Myc-induced formation of the apoptotic cell islands, whereas the mechanism for the c-Myc-induced cell proliferation occurs within the proliferating cells without involving their neighboring cells.

Another morphological feature of c-*myc* and *tgfα*/c-*myc* tumors is that they contain little stromal tissue. Stromal cells and matrix, as well as blood capillaries are all much fewer, compared with the mammary tumors from parous MT-*tgfα* transgenic mice or from other experimental mice reported in the literature. This difference in the abundance of stroma has already become prominent in the hyperplastic mammary tissue before the appearance of frank tumors. Expression of c-Myc in lung cancer cells has recently been shown to suppress the expression of vascular epithelial growth factor (VEGF) (Barr *et al.* 2000). It is thus conceivable that in the c-*myc* and *tgfα*/c-*myc* mammary tissue and mammary tumors, the low abundance of stroma in general and of blood capillaries in particular may, in part, be related to the suppression of VEGF by c-Myc. This trait may also be partially responsible for the rareness of distant metastases of these tumors.

Possible multiple stages of c-Myc-induced mammary carcinogenesis

In principle, carcinogenesis is a multi-staged process of initiation, promotion, and progression; each of these stages also consists of multiple steps. However, none of the current animal models of mammary carcinogenesis has been observed to manifest a clear multi-step nature, except for the noticeable hyperplastic lesions prior to tumor formation. In our studies of the c-*myc* transgenic model, we noticed that in some of the relatively larger (>1 cm in diameter) mammary tumors, there are focal areas of tumor cells that are both hematoxylin (H)- and eosin (E)-phobic on routine H-E stained sections (Liao *et al.* 2000). In these focal lesions, the number of apoptotic cells are much fewer, while the number of proliferating cells are much greater compared with the surrounding tumor areas. Although these foci show a clear boundary of demarcation from surrounding tumor areas, they are not encompassed by connective tissue capsules. Usually, some portion of each focus exhibits infiltration into the adjacent tumor areas, a typical feature of invasive growth. All these morphological properties of the 'tumor-within-a-tumor' foci in c-*myc* tumors suggest that they may belong to a tumor phenotype that is more aggressive than their adjacent tumor area, and may thus represent a second step of tumor progression.

A question then raised is whether the appearance of these 'tumor-within-a-tumor' foci is unique for the c-*myc* transgenic model or, rather, is a common phenomenon among experimental models of mammary carcinogenesis. In our study of the tumors from MT-*tgfα*/MMTV-c-*myc* double transgenic mice, we have not observed such foci. However, the rates of cell proliferation and apoptosis in the entire tumors were comparable to those in the foci of the c-*myc* tumors. Thus, it seems that the second stage of tumor progression may be circumvented in this double transgenic model, and the multiple steps of progression may have been completed very early, before the transformed cells develop into a frank tumor. However, it cannot be excluded that this second step of progression may still appear in other single or double transgenic models of mouse mammary carcinogenesis.

c-Myc and sex hormones

Estrogens play complex roles in mammary gland development and carcinogenesis. The roles of estrogens in

cell proliferation in their target organs are presumably exerted, in part, via a set of estrogen-responsive genes, including c-*fos*, c-*jun*, and c-*myc* (Schuchard *et al.* 1993, Hyder *et al.* 1994). Many *in vitro* studies and some *in vivo* experiments (mainly in uterine tissue) have shown that expression of c-*myc* mRNA is induced by treatment of estrogens (Schuchard *et al.* 1993, Shiu *et al.* 1993, Hyder *et al.* 1994). A 116 bp DNA sequence, which does not contain the canonical estrogen-responsive-element (ERE), in the promoter region of the human c-*myc* gene is responsible for the transcriptional activation by estrogens (Dubik & Shiu 1992). It is likely that activation of the c-*myc* gene by estrogens requires binding of some ER-associated proteins to ER. It remains obscure if and how estrogen-ER signaling regulates c-*myc* expression in human breast tumors (Miller *et al.* 1993), as several reports (but not all) show that overexpression and/or amplification of c-*myc* occurs preferentially in ER-negative tumors.

ER-positive breast tumors from patients who have received tamoxifen treatment show a decreased level of c-*myc* mRNA, compared with their counterparts without tamoxifen treatment (Le, X *et al.* 1991). Similar inhibition of c-*myc* expression by antiestrogen has also been observed in ER-positive T–47D and MCF–7 cells (Wong & Murphy 1991, Tsai *et al.* 1997). These results suggest that tamoxifen antagonizes the effects of estrogens on c-*myc* expression both *in vivo* and *in vitro*. However, treatment with tamoxifen has also been shown to induce apoptosis of both ER-negative and ER-positive breast cancer cells, in association with an induction of c-*myc* expression (Kang *et al.* 1996). Moreover, tamoxifen also inhibits the growth of MCF–7 tumor growth in nude mice, in association with an induction of c-*myc* expression (Santoni-Rugiu *et al.* 1998). The role of increased c-Myc in this latter case was considered, without direct proof, to be related to tumor cell differentiation.

A strong, synergistic role of androgen in estrogen-induced lieomyomas and sarcomas in the uterus and scent gland in hamsters has been known for thirty years (Kirkman & Algard 1970*a,b*, Kirkman 1972, Dodge *et al.* 1976). Accumulating epidemiological data also suggest that elevated androgens, mainly testosterone secreted from ovaries, may contribute to the development of breast cancer in women (Berrino *et al.* 1996, Lopez-Otin & Diamandis 1998, Cauley *et al.* 1999, Yu *et al.* 2000). In an attempt to induce prostate cancer with both 17β-estradiol and testosterone propionate, Liao *et al.* (1998) unexpectedly found that all male rats receiving both hormones develop invasive mammary cancer at a time point when the tumors had not yet developed in the rats receiving only estrogen. Soon afterwards Xie *et al.* (1999*a,b*) also reported similar findings in the female Noble rats. These data are the first experimental evidence demonstrating a synergistic effect of testosterone and estrogen in the induction of mammary cancer, thus raising a concern on the use of androgens in certain hormone replacement therapy in woman patients (Bartlik & Kaplan 1999, Basson 1999, Hoeger & Guzick 1999).

There is currently no clue as to how testosterone plays a role in carcinogenesis of mammary gland and uterus. It is possible that testosterone may be converted to estrogen by aromatase and thus function as increased estrogen (Henderson & Feigelson 2000). However, since normal mammary glands and most breast tumors in both human and rodents express significant amounts of androgen receptor (AR) (Wilson & McPhaul 1996, Liao *et al.* 1998), it is also possible that testosterone may bind directly to the AR and perform an as yet undefined role in promotion of carcinogenesis. Androgen can stimulate or inhibit the transcription of AR (termed autoregulation), depending on the cell-type (Kokontis *et al.* 1994, Asadi & Sharifi 1995, Umekita *et al.* 1996, Kokontis & Liao 1999). C-Myc/Max heterodimer has been shown to bind to a Myc E box element in the AR gene and participate in the autoregulation of AR by AR (Grad *et al.* 1999). On the other hand, it has been shown that androgen induces c-*myc* expression to promote proliferation of prostate cancer cells in culture (Kokontis *et al.* 1994, Umekita *et al.* 1996, Kokontis & Liao 1999), although it is unclear if similar effects also appear in other AR-expressing tissues. Thus, it cannot be excluded that in mammary gland and breast cancer, c-*myc* expression is subjected to the transcriptional regulation not only by estrogen but also by elevated androgen. The increased c-Myc may participate in autoregulation of AR expression to control the as yet undefined role of androgen in breast cancer development.

Progesterone influences differentiation, proliferation, and other functions of the mammary gland by mechanisms that are more complicated and less understood (Clarke & Sutherland 1990). Combined treatment with both progesterone and estrogen has been shown to have a stronger effect than estrogen alone on the induction of mammary tumors in male rats (Hannouche *et al.* 1982). The question as to whether progesterone has an impact on breast cancer development in humans is an important one, because progesterone is used widely in oral contraceptives and in hormone replacement therapy for postmenopausal women. Treatment of cultured breast cancer cells (MCF-7 and T47-D) with progestin results in transient acceleration of the G1 phase, followed by cell cycle arrest and growth inhibition (Musgrove *et al.* 1991, 1998). These data led to an hypothesis that the action of progesterone is to accelerate the replication of the cells already progressing through G1, which are then arrested early in G1 after completing a round of cell replication (Musgrove *et al.* 1991, 1998). The c-Myc protein is considered to be the mediator in this transient growth stimulation followed by growth inhibition (McMahon *et al.* 1998). Indeed, expression of c-*myc* mRNA is rapidly but transiently induced by progestin treatment, whereas

relatively long-term treatment of progestin results in suppression of its expression (Musgrove *et al.* 1991, Wong & Murphy 1991). Consistent with this suppressive role, overexpression and/or amplification of the c-*myc* gene has been observed to occur preferentially in PR-negative breast cancer cases (Adnane *et al.* 1989, Berns *et al.* 1992*c*).

The promoter of the human c-*myc* gene contains a 15-bp sequence with homology to the progesterone response element (PRE) (Moore *et al.* 1997). Binding of PR to this sequence results in activation of the reporter gene, as studied in a CAT assay. However, it is still unclear whether this PRE-like sequence is involved in the transient stimulation and then inhibition of expression of the c-*myc* gene by progesterone in breast cancer cells. In avian oviducts, PR activates expression of the c-*myc* gene (Fink *et al.* 1988), which is mediated by the interaction of certain nuclear matrix-associated steroid receptor binding proteins (Barrett *et al.* 2000). It seems that the effects of progesterone on PR-responsive cells are cell-type specific, and that the specificity may be related to the mediation of some PR-associated proteins.

Prolactin is both a mitogen and a differentiating agent in the mammary gland. In several rodent models prolactin has been shown to have potent, promotive effects on mammary cancer development (Vonderhaar 1999). A role in human breast cancer has also been suggested, although unproved (Vonderhaar 1998, 1999, Hankinson *et al.* 1999). Prolactin receptors are present in about 70% of human breast cancer biopsies (Clevenger *et al.* 1995). Cultured breast cancer cells respond to prolactin as a mitogen. Prolactin is synthesized by human breast cancer cells, and inhibition of the binding of prolactin to its receptors inhibits the cell growth (Vonderhaar 1998). Prolactin can cause a dose-dependent increase in the levels of c-*myc* mRNA in hepatocytes both *in vivo* and *in vitro* (Crowe *et al.* 1991, Zabala & Garcia-Ruiz 1989). However, how prolactin affects c-*myc* expression in the reproductive organs and tissues is unknown. Since activation of the prolactin-prolactin receptor pathway simulates the Sos/Ras and Vav/Rac signaling cascades in several breast cancer cell lines (Vonderhaar 1998), it is conceivable that c-Myc is also mediated in certain functions of prolactin in mammary gland.

Relevance of the c-*myc* gene to breast cancer prognosis and therapy

Several reports have shown an association of c-*myc* gene amplification with a poor prognosis of breast cancer (Berns *et al.* 1992*c,d*, 1996, Borg *et al.* 1992, Roux-Dosseto *et al.* 1992, Scorilas *et al.* 1999), whereas many other studies do not find such a correlation (Table 1; Deming *et al.* 2000). Reports on the prognostic value of overexpression of c-*myc* mRNA or protein are not only inconsistent but also conflicting (Table 1). While many other studies do not find any association between c-*myc* expression and prognosis (Mizukami *et al.* 1991, Spaventi *et al.* 1994), several investigations find that a higher expression level correlates with a poorer outcome (Guerin *et al.* 1988, Pertschuk *et al.* 1993, Mimori *et al.* 1998). However, a recent study shows that higher c-*myc* mRNA levels in breast cancer are correlated with better survival (Bieche *et al.* 1999). This conclusion is consistent with the earlier immunohistochemical studies on protein levels, but probably is correct only for the axillary lymph node negative tumors (Pietilainen *et al.* 1995). Similar findings that higher levels of c-*myc* expression reflect better prognosis have also been reported for patients with other types of malignancy, such as testicular cancer (Watson *et al.* 1986), colorectal cancer (Smith & Goh 1996), uveal melanoma (Chana *et al.* 1998, 1999), and probably also ovarian cancer (Diebold *et al.* 1996, Tanner *et al.* 1998). Many other studies that do not involve analysis of survival also show that higher levels of c-Myc proteins are discerned in better differentiated cancer in testis (Sikora *et al.* 1985), colon (Sikora *et al.* 1987, Watson *et al.* 1987*b*, Royds *et al.* 1992), ovary (Watson *et al.* 1987a, Polacarz *et al.* 1989), and bile ducts (Voravud *et al.* 1989), which is also likely to be coupled with a better outcome. Moreover, ectopic expression of c-*myc* in lung cancer cell lines has recently been shown to suppress tumor development from cells injected into nude mice (Barr *et al.* 2000). Several studies show that benign breast lesions such as fibroadenomas and fibrocystic disease express c-Myc at levels as high as seen in breast cancer (Whittaker *et al.* 1986, Spandidos *et al.* 1987). This property leads to a consideration that c-Myc may be involved in the early development of the cancer and could be used as a marker for the pre-malignancy or for the risk of the cancer.

The controversial prognostic implications for c-*myc* overexpression should not be surprising, for a few reasons. First, c-Myc proteins may direct cells to proliferation, differentiation, or apoptosis; in the two latter cases the c-Myc level may correlate with a better outcome. Secondly, c-Myc may suppress expression of VEGF in the tumor (Barr *et al.* 2000), which may also be associated with a favorable prognosis. Thirdly, as mentioned above, the c-*myc* gene product engenders different proteins that may have different, and even opposite functions, and it is currently unknown which of the c-Myc protein isoforms is expressed in which *in vivo* situation. Moreover, c-*myc* may induce cell proliferation, but proliferating cells are usually more sensitive to chemotherapy. Indeed, colonic cancer with low levels of c-*myc* gene amplification has been reported to respond to adjuvant chemotherapy much better than that without the gene amplification (Augenlicht *et al.* 1997). This may explain why higher c-*myc* expression levels are correlated on the one hand with larger sizes of breast tumors but on the other hand with a better survival (Bieche *et al.* 1999).

In our c-*myc* transgenic mouse model, we have observed that c-*myc* expression in the transgenic mammary tumors is actually attenuated in the highly proliferating, less apoptotic tumor foci (Liao *et al.* 2000). Interestingly, these specific foci show high expression levels of cyclins D1 and E, whereas their adjacent tumor areas do not express these cyclins. Hyperplastic mammary glands in the c-*myc* mice do not show expression of these cyclins either. Since *in vitro* studies have demonstrated that c-Myc can suppress expression of cyclin D1, it is likely that the decrease in c-Myc expression in the foci enables the overexpression of these cyclins. Thus, one possible explanation for the inverse correlation between c-*myc* level and prognosis is that rises in both G1 cyclins, D1 and E, may lead to an unfavorable outcome (Scott & Walker 1997, Wilcken *et al.* 1997, Nielsen *et al.* 1998, Lin *et al.* 2000), whereas high levels of c-Myc prevent their surges.

However, why do high levels of c-Myc associate with an unfavorable outcome in other cases? Attempting to answer this question, we compared the *tgfα*, c-*myc*, and *tgfα*/c-*myc* transgenic models. This is because not only cyclins D1 and E (Jansen-Durr *et al.* 1993, Gillett *et al.* 1996, Nielsen *et al.* 1996, Sasano *et al.* 1997, Trudel *et al.* 1997), but also TGFα and several other growth factors (Panico *et al.* 1996) or their receptors (Dickson & Lippman 1995) that are known to be survival factors for c-Myc-overexpressing cells (Hoffman & Liebermann 1998, Prendergast 1999), have all been reported to be overexpressed in most cases of human breast cancer (Auvinen *et al.* 1996). In contrast to the suppressive effects of c-Myc, TGFα may induce cyclin D1, as suggested by the observation that many cyclin D1-positive cells appear in the hyperplastic mammary epithelium from *tgfα* transgenic mice (Liao *et al.* 2000). In *tgfα*/c-*myc* double transgenic tumors, many cyclin D1-positive cells also appear, but they are spread randomly throughout the whole tumors, without forming any specific foci or showing reciprocal relation to the c-*myc* expression (Liao *et al.* 2000). Collectively, these data suggest that in the double transgenic model, TGFα induces expression of cyclin D1, which overrides the suppression by c-Myc. Cyclin E is also overexpressed in a way similar to the expression of cyclin D1 in the *tgfα*/c-*myc* tumors (Liao *et al.* 2000). Thus, three types of tumor tissue, i.e. the major tumor areas of c-*myc* tumors, the foci in c-*myc* tumors, and the *tgfα*/c-*myc* tumors, manifest different patterns of relationship between c-Myc and G1 cyclins, respectively: (1) c-Myc overexpression without rises in G1 cyclins, (2) loss of c-*myc* expression with overexpression of G1 cyclins, and (3) high expression levels of both c-Myc and G1 cyclins under the condition of a concomitant increase in TGFα. As described above, the latter two patterns are associated with a higher proliferative rate and a lower apoptotic rate, compared with the former one.

These three patterns of relationships among c-Myc, G1 cyclins, and growth factors provide one possible explanation for the dual prognostic values of c-*myc* overexpression. It seems that constantly high levels of c-Myc as seen in the major c-*myc* tumor areas tend to commit the cells to apoptosis, unless this trend is converted to that of proliferation by one of two conditions: (1) the c-Myc level drops to allow G1 cyclins to increase, as seen in the c-*myc* tumor foci, or (2) the level of TGFα (or other survival factor) is concomitantly increased to rescue G1 cyclins from suppression by c-Myc and to cause their induction. If a breast cancer manifests constant overexpression of c-Myc without concomitant increase in TGFα (or other survival/proliferation-promoting growth factors) or G1 cyclins, the tumor may undergo apoptosis and be more sensitive to apoptotic stimuli or to chemotherapy, which may be reflected in a better prognosis. Under this situation, antagonism of c-*myc* expression, such as by utilization of c-*myc* antisense oligonucleotides that has been proposed as a strategy for gene therapy of cancer, may actually prevent apoptosis of the tumor cells and may risk a rise in G1 cyclins and development of a more aggressive tumor phenotype. On the other hand, should the tumor show a high level of c-Myc with concomitant increase in growth factors or G1 cyclins, it may be more aggressive. In this latter case, neutralization of the cyclins and/or survival factors, alone or together with antagonism of expression of c-*myc* gene by factors such as antisense treatment, may be a more appropriate way to commit the cells to apoptosis. Since a cancer usually contains heterogeneous tumor cell populations, all these patterns of relationship among c-Myc, cyclins and growth factors are likely to exist concomitantly. Thus, the challenge is that one single therapeutic regimen may kill some cancer cells while allowing some other cells to evolve to more aggressive forms.

Special biological properties of the mammary gland

As already pointed out by many other investigators, reports on the roles of c-Myc in proliferation, apoptosis, differentiation, and growth of a cell are often conflicting. Part of the reason may be that the function of c-Myc is cell type-specific and dependent on experimental conditions. However, most of our understanding of the mechanisms for the functions of c-Myc derives from experiments with *in vitro* systems and with fibroblasts or cells of hemopoietic or lymphpoietic origins. These types of cells differ from epithelium in many respects.

There are, indeed, some studies on c-Myc using epithelial tissues that are not primary targets of sex steroids and which differ greatly from mammary gland, such as liver and kidney. Growth stimuli to liver can be divided into the compensatory type, which is associated with significant cell loss, and the non-compensatory type (Coni *et al.* 1993,

Columbano & Shinozuka 1996). In response to the compensatory-growth stimulus such as partial hepatectomy or treatment with necrogenic agents, hepatocytes quickly enter the cell cycle from the quiescent G0 phase, characterized by increased expression of immediate-early genes such as c-*fos*, c-*jun* and c-*myc*. The cells proliferate to restore the normal number of hepatocytes and normal size of liver, followed by cessation of proliferation, probably triggered by contact-inhibition between hepatocytes. Non-compensatory growth stimuli such as treatment with phenobarbitol, estrogen, growth factors, or many other agents that are not necrogenic to the liver, also induce hepatocytes to proliferate, leading to the enlargement of the liver. However, the proliferated cells tend to undergo apoptosis, especially after withdrawal of the growth stimulus, probably because the liver must reduce its size to normal. Ductal epithelial cells of the kidney normally rest at the G1 phase, and are refractory to various proliferating stimuli (Norman *et al.* 1988). Unirenalectomy, which causes a 50% loss of kidney tissue, mainly causes hypertropy of the remnant kidney, i.e. growth in the cell size of the ductal epithelium, with little cell proliferation, although c-*myc* and other immediate-early genes are activated by the operation (Norman *et al.* 1988).

Unlike liver, kidney, and other epithelium-dominant organs, the mammary gland is not fully developed until the females deliver and nurse their offspring. After weaning, most of the developed glands will undergo atropy, mainly via apoptotic pathways. More unusually, the glands are embedded inside a fat pad before their growth during development or their induction by various stimuli. The meaning of this property may be much more profound than scientists have realized. For instance, the glands may proliferate freely in response to growth stimulation, extending into the fat pad with no strict size control, although there may still exist contact-inhibition among neighboring epithelial cells. If so, there may not exist the non-compensatory type of growth stimuli for the mammary gland; its proliferation may not be coupled with a tendency for apoptosis to limit the gland size, as may be the case in the liver. The apoptotic process after weaning may differ in its mechanism from the apoptosis of hepatocytes after withdrawal of non-compensatory stimulus. Moreover, the property of 'growth within a fat pad' may also make the stromal-epithelial interaction more involved than in other organs. Other unanswered questions regarding mammary gland biology include the determination of which cell cycle stage (G0 or G1) the majority of mammary epithelial cells normally rest. What is clear is that mammary gland epithelium has its distinct biological features; special precautions should be taken when applying knowledge from studies on other non-epithelial and epithelial systems to the mammary gland.

Perspectives

Data pertaining to several fundamental questions on the functions of c-Myc are still very confusing. For instance, do c-Myc proteins play a role in all early and advanced stages of the carcinogenic process? Are tumor cells in a cancer that express high levels of c-Myc proteins more malignant or more benign? Can overexpression of c-Myc in a spontaneously occurring tumor trigger an apoptotic process as can ectopically expressed c-Myc? What does a cytoplasmic localization of c-Myc protein suggest to us? Superficially, the answers seem to be that c-Myc should be a nuclear protein, as it is a transcription factor, and that it should contribute to all stages of the carcinogenic process to make a normal cell progressively more malignant (Garte 1993), as it may cause genetic instability. Thus, a positive immunohistochemical staining should be observed in the nucleus of tumor cells and should reflect an unfavorable outcome.

However, data on the c-Myc expression in various types of human malignancy and data from our transgenic models are often inconsistent with this line of inference. We now consider that, while the above inference may still be correct on many occasions, under certain circumstances c-Myc protein may play a 'hit-and-run' game during the whole carcinogenic process. In these special, as yet undefined situations, c-Myc may just contribute to the tumor onset and early growth. Once a tumor is formed and has grown to a certain size, high levels of c-Myc may cause some additional effects that are unfavorable for the survival and continuous growth of the tumor. These effects may include the tendency to apoptosis or the high sensitivity to apoptotic factors, as well as the inhibition of VEGF, G1 cyclins, Her2/neu, etc. Accumulating experimental evidence is now suggesting that c-Myc may be an important transcriptional repressor. Some of the genes suppressed by c-Myc may be required for the survival or growth of the tumor cells in a hostile environment, such as the hypoxia that often occurs when a tumor becomes large and lacks a sufficient blood supply. Under these circumstances, certain c-Myc-signaling pathways may be shut off. Examples of this are the attenuation of c-*myc* expression and the prevention of its entry into the nucleus. These lines of thinking need further validation by experimental and clinical studies.

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Appendix III

Atypical apoptosis in MMTV-c-*myc* transgenic mouse mammary tumors

Dezhong Joshua Liao[1,2], Gloria Chepko[1,2], and Robert B. Dickson[1,2,3]

[1]Lombardi Cancer Center, [2]Department of Oncology, and [3]Department of Cell Biology, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, DC 20007

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Correspondence:

Dr. Robert B. Dickson, Research Building, W417, 3970 Reservoir Road, NW, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007

Tel: (202)687-3337

Fax: (202)687-7505

Email: DicksonR@gunet.georgetown.edu

Abstract

Enforced expression of c-*myc* has been shown to serve as an apoptotic stimulus in cultured cells, in the absence of survival factors. Prior studies have also demonstrated that tissues expressing the c-*myc* transgene display a large number of dead cells. In the present study, we found that MMTV-c-*myc*-transgenic mouse mammary tumor cells exhibited mitochondrial malformation, characterized by a primarily amorphous matrix, with very few cristae. Mitochondria were also frequently degenerated. Cytochrome c expression was much lower in the majority of MMTV-c-*myc* mammary tumor cells, compared to adjacent, c-*myc*-silenced tumor foci. In the majority of the tumor areas, there were many dying and dead cells organized in clusters, termed dead cell islands. These cells exhibited shrinkage, TUNEL positive staining, nuclear localization of apoptosis-inducing factor (AIF), but a lack of typical apoptotic morphology, such as nuclear condensation and formation of cell membrane blebs and apoptotic bodies. Many macrophages were detected infiltrating into these dead cell islands and engulfing the dying or dead tumor cells. Disruption of tumor cells was rare, but disrupted macrophages were common. These morphologic features suggest that the atypical cell death in c-*myc* transgenic mammary tumor tissue may be related to malformed and degenerated mitochondria, possibly leading to energy deficiency, and to the early involvement of macrophages engulfing the dying cells.

Introduction

A review of the literature on the differences between apoptosis and other types of cell death, mainly necrosis, is currently very confusing, mainly due to the lack of a strict definition of apoptosis (Levin *et al.* 1999; Lockshin 1999; Majno and Joris 1999; Afford and Randhawa 2000; Johnson 2000; Nicotera *et al.* 1999; Columbano 1995; Kroemer *et al.* 1998; Fiers *et al.* 1999). As summarized by Blagosklonny (Blagosklonny 2000), apoptosis has been commonly described, but not precisely defined from four different points of view: 1) programmed cell death, 2) a combination of morphological features, such as nuclear fragmentation, chromatin condensation, DNA degradation, cell shrinkage, and membrane blebbing; 3) an active process of dying that requires energy and *de novo* gene expression; 4) cell death which is non-necrotic. The last point of view may be the main source of confusion, because the term necrosis is mainly used to refer to a tissue and thus has little relevance and application to cell culture where apoptosis is mostly studied (Blagosklonny 2000). According to the original definition of apoptosis *in vivo* (Kerr *et al.* 1972), a cell either in the process of dying or dead, via an apoptotic mechanism, will be phagocytosed by its neighboring cells or macrophages, such that neither antigenic nor toxic components are released to elicit an inflammatory response that often results in scar formation. As distinct from this *in vivo* process, apoptosis of cultured cells does not involve clearance by macrophages, and thus differs from tissue apoptosis at least in the last steps. If the rate of apoptosis in a tissue exceeds the rate of clearance of these cells by macrophages, it is possible that inflammatory and toxic processes could result, leading to a secondary necrosis. This type of situation would be artificially induced by enforced death signaling, such as might occur in a model system

driven by a cell death-associated gene (i.e. c-*myc*) or in human tumors treated with certain types of chemotherapy (Aoyagi & Dickson 1999).

Because it is difficult and even impossible to distinguish apoptosis from necrosis in many circumstances, programmed cell death, a more general nomenclature, is often used in the description of the death. Both programmed cell death in general, and apoptosis in particular, have been shown to occur via either caspase-dependent or caspase-independent mechanisms (Borner & Monney 1999; Elliott *et al.* 2000; Johnson 2000; Chan and Mattson 1999; Hotti *et al.* 2000; Vier *et al.* 1999; Kolenko *et al.* 1999). While more studies continue to describe the biochemical differences between these two pathways, very few data have been presented in the literature to morphologically contrast these two pathways, especially in tissues. This aspect is of importance, since morphologic features are still the major criteria used to distinguish different types of cell death.

c-Myc oncoproteins have been shown to play dual roles in cell proliferation and cell death (Conzen *et al.* 2000; Dang *et al.* 1999; Hoffman and Liebermann 1998), similar to many other oncoproteins, such as cyclin D1, E2F1, Ras, and E1A (Guo and Hay 1999; Duelli and Lazebnik 2000; Chi *et al.* 1999). The mechanisms for c-*myc*-triggered cell death have been addressed extensively in the recent years (Prendergast 1999; Packham & Cleveland 1995; Alarcon *et al.* 1996; Thompson 1998; Borner and Monney 1999), but mostly with cultured cells. Both caspase-dependent and caspase-independent pathways have been implicated in the mechanisms for the apoptotic effects of c-Myc in cell culture (Thompson 1998; Hotti *et al.* 2000; Juin *et al.* 1999; Prendergast 1999; McCarthy *et al.* 1997). Far fewer studies have addressed the mechanisms of cell death in c-*myc* expressing tissues.

In different transgenic mouse models, c-*myc* has been targeted to the mammary gland (Stewart *et al.* 1984), liver (Sandgren *et al.* 1989), lymphocytes (Felsher and Bishop 1999; Pelengaris *et al.* 2000), and renal ducts (Trudel *et al.* 1997). Mammary adenocarcinomas, hepatocarcinomas, lymphomas, and polycystic kidneys develop, respectively, in these animals as the consequences of the expression of the transgene. All of these cancers or benign lesions show many dead cells, which are commonly described as a result of apoptosis. Moreover, the c-*myc* transgene has also been targeted to the nervous system (Jensen *et al.* 1998) and the pancreas (Pelengaris *et al.* 2000); pronounced cell death is also discerned in these transgenic tissues. However, in most of these studies (Felsher and Bishop 1999; Sandgren *et al.* 1989; Hundley *et al.* 1997; Pelengaris *et al.* 2000; Stewart *et al.* 1984; Trudel *et al.* 1997; Christensen *et al.* 1999; Sanders and Thorgeirsson 1999), including our own (Amundadottir *et al.* 1996; McCormack *et al.* 1998; Liao *et al.* 2000a), the term "apoptosis" has not been applied based on solid morphologic or biochemical evidence; a positive staining of TUNEL is the only indication in most cases. This may be because the concept that c-Myc induces apoptosis has been well established in the past in cell culture systems (Shi *et al.* 1992; Evan *et al.* 1992) where it has been well accepted, and this description is simply extended to c-*myc*-expressing tissue systems *in vivo*.

In the present study, we described for the first time the detailed morphology of cell death in a tissue triggered by enforced expression of c-*myc*. It was found that mammary tumor cells from c-*myc* transgenic mice exhibited malformation and degeneration of their mitochondria, in association with a very low level of cytochrome c expression. The dying cells manifested shrinkage, DNA breakage as indicated by a positive TUNEL staining,

and nuclear localization of apoptosis inducing factor (AIF), but in the absence of typical apoptotic morphology, including nuclear condensation and formation of cell membrane blebs and apoptotic bodies. In addition, the dying process attracted pronounced infiltration of macrophages, without an inflammatory response. Disruption of dead tumor cells *per se* was rare, but disruption of macrophages was common. These morphologic features indicate that the cell death in the c-*myc* transgenic mammary tumor tissue may not be described as a typical apoptosis.

Materials and Methods

Tissue collections. Tumor tissue materials of MMTV-c-*myc* transgenic mice used for this study were from our previous report (Liao *et al.* 2000a), with additional animal experiments that were carried out similarly. At the sacrifice of the animals, mammary tumors were isolated. Part of the tumor tissue was sliced into thin pieces and immediately fixed with 10% formalin in PBS (pH 7.4) overnight at 4 ^{0}C. The tissue was subjected to a quick (6 hours) procedure of tissue processing and was embedded in paraffin. In total, 30 paraffin-embedded tumors were sectioned (5 μm) and stained with hematoxylin and eosin (HE staining). Other pieces of tumor were first frozen in liquid nitrogen and then stored at –80 ^{0}C for further molecular analyses of the tumors.

Toluidine blue staining and electron microscopy. At sacrifice of the animals, six tumors were prepared for electron microscopy. For this purpose, fresh tissue biopsies collected from five different places in each tumor were chopped into about 1 mm^3 pieces and then immediately fixed with 3% glutaraldehyde in PBS (pH 7.2) overnight at 4 ^{0}C. The glutaraldehyde-fixed tumor tissues were post-fixed with 1% osmium tetroxide

(Sigma Chemical Company, St. Louis, MO) for 1 hour and washed with distilled water 3 times (10 minutes each). The tissues were stained with 2% uranyl acetate (Sigma Chemical Company, St. Louis, MO) for 30 minutes in the dark and washed with distilled water 3 times, followed by dehydration with graded ethanols and then propylene oxide. The tissues were incubated in a mixture of propylene oxide and Spurr resin overnight and then in Spurr for 6 hours. The tissues were embedded with Spurr and cured in an oven at 65 ^{0}C overnight. Semi-thin sections (1um) were cut on Reichert Ultracut microtome and stained with 1% toluidine blue in 1% Borax. Ultrathin sections (70-80 nm) were cut on the same microtome, mounted on 200 mesh grids and stained with lead citrate. Grids with stained sections were viewed on JEOL 1200EX electron transmission microscope and pictures were taken.

TUNEL assay. The terminal deoxynucleotidyl transferase (TdT) mediated digoxigenin-dUTP nick end labeling (TUNEL) method was carried out using a kit from Trevigen Inc., Gaithersburg, M.D, as described previously (Liao *et al.* 2000a). Paraffin sections (5 μm) were dewaxed, rehydrated, treated with protease K, and blocked with H_2O_2, according to the manufacturer's instructions. After labeling with TdT and biotin-labeled dNTP, the sections were incubated with peroxidase-conjugated Streptavidin. The signal was visualized by exposure to diaminobenzidine and H_2O_2, followed by counter-staining with hematoxylin.

Immunohistochemistry. Immunohistochemical staining was performed using an avidin-biotin complex (ABC) method described previously (Liao *et al.* 2000a). Paraffin sections were deparaffinized and blocked with 3% peroxide. Antigens were retrieved by heating in a microwave oven in 50 mM citrate buffer, pH 6.0, after boiling for 8 minutes.

After blocking with 6% normal goat or horse serum, the sections were incubated with the primary antibody for 2 hours, followed by 1 hour incubation with a second antibody conjugated with biotin (Vector Laboratories Inc., Burlingame, CA). The sections were then incubated with peroxidase-conjugated avidin (Dako, Corporation, Carpinteria, CA) for 30 minutes, followed by color development with diaminobenzidine and peroxide. All procedures were carried out at room temperature. The primary antibodies used were rabbit polyclonal cytochrome c antibody (H-104), goat polyclonal CD68 antibody (M-20), and goat polyclonal antibody (D-20) against apoptosis inducing factor (AIF), all from Santa Cruz Biotechnology Inc., CA. To control for signal specificity, serial sections were made from four tumors and were subjected to the same staining procedure, with a normal rabbit or goat IgG to replace the respective primary antibody. This control staining did not give rise to a signal, demonstrating the specificity of the signal given by the primary antibodies.

Western blot analysis. Frozen tumor tissues were homogenized with a polytron in a lysate buffer containing (Liao *et al.* 2000a, 2000b): 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.5 mM Na_3V0_4, 20 mM sodium pyrophosphate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM DTT. The tissue lysates were transferred to a B-type glass homogenizer, homogenized again, and centrifuged at 12,000 r.p.m. for 20 min at 4 ^{0}C. The supernatants were collected, and their protein concentrations measured with BCA reagents (Pierce, Rockford, IL). Protein aliquots (70 μg per lane) were electro-fractionated on SDS-PAGE. Roughly equal loading was confirmed by staining the gel with Coomassie blue. The proteins were transferred onto a PVDF membrane and probed with primary antibody for 2 h, followed

by incubation with a peroxidase-conjugated second antibody for 1.5 h. The signal was visualized with chemiluminescent substrates (Pierce, Rockford, IL) and autography. The primary antibodies used in western blot analyses were the same as used for immunohistochemical staining.

Results

Cell death in HE-stained sections

In a previous study, we had reported that some large, c-*myc* transgenic mammary tumors, at advanced stages, developed focal lesions that we termed "foci". These "tumor-within-a-tumor" focal lesions were morphologically distinguished from their surrounding tumor cells (fig 1 in Liao *et al.* 2000a). In the present study, we found that there are two major types of foci, according to their morphologies. One was a solid type, in which tumor cells constituted a solid, nodular structure, as was shown in the previous report (fig 1 in Liao *et al.* 2000a). The second type of focus we now report on is glandular, in which tumor cells form glands of various sizes (fig 1A). Occasionally, the foci also exhibited a mixture of morphology of these two types (fig 1B). All of these foci shared several key properties, including refractoriness to hematoxylin staining, decrease in c-*myc* expression, induction of cyclins D1 and E, increase in proliferation, and decrease in programmed cell death, when compared to their surrounding tumor areas (Liao *et al.* 2000a; Liao and Dickson 2000).

The c-*myc* transgenic mammary tumors exhibited a large number of dead cells (Liao *et al.* 2000a), which were usually organized in clusters (fig 1C). Individual dead cells also appeared, but with less frequency. There was always a space between a cluster

of dead cells and the surrounding viable tumor cells (fig 1A and 1C). This space was likely to result from shrinkage of the dying or dead cells. The appearance of this space made each cluster of dead cells look like an island in the c-*myc* tumor; therefore the clusters were termed "apoptotic cell islands" in our previous publication (Liao and Dickson 2000). The reason for terming the dead cells "apoptotic cells" was primarily because their positive TUNEL staining (Amundadottir *et al.* 1996; Liao *et al.* 2000a). In the present study, the clusters of dead cells are renamed as "dead cell islands" (fig. 1A and 1C), due to their atypical morphology, as discussed below. Moreover, necrotic areas were also observed in c-*myc* mammary tumors, as presented in our prior report (Waikel *et al.* 1999). Since these necrotic areas are common features of tumor tissues in general, we will not consider them further in this study.

Previously, we reported that dead cell islands were basically not seen in the solid type of focus, in strong contrast to their abundance in the major tumor areas (Liao *et al.* 2000a). In the present study, we further report that few dead cell islands appeared in the glandular type of foci (fig 1A). However, there was often a cluster of dead cells inside the lumen-like cavities of the tumor glands (fig 1A and 1D). Although these clusters of cells also manifested an "island" morphology, the spaces between the cell clusters and the surrounding tumor cells were actually the lumen-like features of the tumor glands, and thus different from the space surrounding the dead cell islands (which was likely to be a result of cell shrinkage). The dead cells inside the gland cavities resembled necrotic cells more closely than apoptosis in the HE stained sections (fig 1D), and were morphologically different from dead cell islands.

Formation of dead cell islands seemed to begin with shrinkage occurring in several tumor cells simultaneously (fig 1E), making the cells separate from their neighboring tumor cells. At this stage, the cells still showed a morphology similar to their neighboring cells (fig 1E). Later in the dying process, nuclear fragmentation, without obvious condensation, appeared as the main alteration in these cells (fig 1F and 1 G). Condensed nuclei were observed only occasionally (fig 1H). Disruption of cell membranes and cytoplasm seemed to be common, leaving much cell debris in the hollow, dead cell islands (fig 1I). Giant cells, containing many small, fragmented, nucleus-like components were frequently observed (fig 1F). Considering the results of toluidine blue staining (see below), these cells might be identified as macrophages with phagosomes containing nuclear fragments, although their true identity could never be certain with HE staining. Blebs of cell membrane, and apoptotic bodies resulting from cell membrane blebbing, were not observed, even when carefully examined under high power in the light microscope.

Hemorrhage was discerned in some of the dead cell islands. However, an obvious inflammatory response, characterized by infiltration of many white blood cells, was ever observed in dead cell islands.

Cell death in toluidine blue-stained sections

In toluidine blue stained semi-thin sections of osmium tetroxide-post-fixed, uranyl acetate in-block-stained tumor blocks, two major types of tumor cells were identified, i.e. dark cells and light cells (fig 2A and 2B). The two types of cells were roughly equal in

number. Each type of cell could dominate in an area, but they were intermixed in other areas (fig 2A and 2B).

In semi-thin section, which brings a higher resolution than in paraffin section, a large number of macrophages was observed in the tumor tissue. They frequently colocalized with dead cell islands (fig 2A and 2B), but sometimes also among viable tumor cells or in the thin stroma (fig 2C). The macrophages were very large in size, usually about five- to eight-fold larger than a viable tumor cell; their huge size made them easily distinguishable from tumor cells. Without exception, all macrophages contained many phagosomes that varied greatly in size; the phagosomes were frequently as large as the nucleus of viable tumor cells (fig 2D). Many phagosomes were intensely stained, while in some other phagosomes, only some components, but not the whole phagosomes, showed the intense staining (fig 2A to 2D). These intensely-stained phagosomes, and the phagosomal components, were likely to be the nuclei or nuclear fragments of dead cells engulfed in macrophages. Consistent with this consideration, these intensely stained phagosomes and phagosomal components appeared more frequently in dead cell islands, where many dead cell nuclei and nuclear fragments were present as seen in HE-stained sections. Moreover, in conflict with the observations from HE-stained sections, few dead tumor cell nuclei were observed to be colocalized with dead cell islands. Thus, many dead cell nuclei observed in the sections stained for HE or TUNEL (see below) might not be the nuclei of dead cells *per se*, but might actually be the nuclei or nuclear fragments of the phagocytosed tumor cells appearing as phagosomes or phagosomal components within macrophages.

Consistent with what was observed in HE stained sections, blebs of cell membrane were not observed in the tumor cells, even though cellular structures were preserved better with glutaradehyde fixation, so any membrane blebbing present should have been discerned. Although disruption of tumor cells was rarely seen, which was in conflict with the observation of cell debris in the dead cell islands in HE-stained sections, disrupted macrophages appeared frequently, releasing phagosomes as well as other cellular components to the intercellular space as cell debris (fig 2E). We interpret this finding as that the cell debris often observed in HE-stained sections may be the result of disrupted macrophages.

TUNEL staining

Many, but not all, nuclei in dead cell islands were positive for TUNEL staining (fig 3A, 3C, and 3D), indicating that DNA breakage occurred in these cells, since the principle for TUNEL staining is DNA end-labeling. Interestingly, in hyperplastic lesions of mammary epithelia adjacent to the tumors, TUNEL positive nuclei appeared individually, but were not organized in clusters (fig 3B). This difference implies that, although cell death in both hyperplastic lesions and tumors might all be triggered by enforced expression of c-*myc*, the mechanisms for cell death in each might still be distinct.

Most TUNEL-positive nuclei in dead cell islands did not show condensed chromatin under high magnification; instead, their chromatin patterns were very similar to that of the nuclei of viable tumor cells, except for the brown TUNEL staining (fig 3A, 3C and 3D). Moreover, in dead cell islands, many nuclei positive for TUNEL were

surrounded by a hollow space, but not by cytoplasm and a cell membrane, although the cytoplasm of the viable tumor cells was clearly discerned by its weak hematoxylin staining. This result indicates that the cytoplasm and cell membrane of these dead cells might have already disrupted and lysed. This process might occur after these tumor cells are engulfed by macrophages, since disruption of tumor cells *per se* was rarely seen in toluidine blue stained sections. Support for this consideration was the observation that many nucleus-like, but smaller than a nucleus, particles were also positive for TUNEL (arrow in fig 3C and 3D). Frequently, these small TUNEL-positive particles were embedded in a cytoplasmic-like component (arrow in fig 3C and 3D), indicating that they were nuclear fragments of a dead tumor cell, engulfed and digested by macrophages. More convincing support for this conclusion was the common observation that groups of positive nuclei were sometimes encapsulated by a membrane (arrowhead in fig 3D).

Electron microscopic (EM) observations

There were also two main types of tumor cells identified under EM, i.e. dark cells and light cells (fig 4A), possibly corresponding to the dark and light cells in toluidine blue stained sections. The nuclei of both types of tumor cells distinctively showed one or more giant nucleolus. Most dark tumor cells showed an undifferentiated and inactive ultrastructure, characterized by electron-dense, amorphous cytoplasmic matrix with very few organelles, such as endoplasmic reticulum and Golgi apparatus. The light cells showed the same absence of cytoplasmic matrix material as described by Chepko and Smith (1997), but they generally contained more organelles than dark tumor cells. Lysosomes, glycogen granules, and lipid droplets were easily discerned in the light tumor

cells (in contrast to dark cells), in addition to larger amount of endoplasmic reticulum and more Golgi complexes. Autophagosomes were also present in some light cells.

For both cell types, the most striking alterations were in the mitochondria. In most tumor cells, the mitochondria consisted mainly of an electron-light (fig 4B and 4C) or electron-dense (fig 4D) amorphous matrix, with absent or very few cristae (fig 4B, 4C and 4D). When the cristae appeared, they were usually dilated to cavities with irregular shapes (arrow in fig 4D). These morphologic features suggest that the mitochondria are malformed in these c-*myc*-induced, c-*myc*-expressing tumor cells. Such malformed mitochondria were frequently degenerated, characterized by the lysis of the amorphous matrix and cristae, leaving a hollow mitochondrial body (fig 4E).

Another distinguishing finding under EM was the confirmation of many macrophages in the tumor tissue, especially in dead cell islands (fig 4F and 4G). Most macrophages were so large and so irregular in shape that it was usually very difficult to take a photo of the whole cell, even under the lowest magnification. All macrophages contained many phagosomes, with various degrees of digested tumor cells, indicating that the macrophages were very active in phagocytosis. The electron density of the phagosomes varied greatly, and it was common that one phagosome showed very high electron density in one part but very low density in the other part (fig 4G, 4I and 4J). The light and dark parts resulted from different organelles and nuclei phagocytosed, and there usually was a clear edge between the light and dark parts (fig 4G and 4I). Peripheral chromatin condensation and nuclear condensation were rarely seen in dying or dead tumor cells, which contrasted to the observation of HE stained sections but was consistent with the observation of the sections stained for toluidine blue and TUNEL. However,

when the electron-dense components resided in the peripheral area or in part of a phagosome, it might make the phagosome resemble a nucleus with peripheral chromatin condensation under the light microscope. Thus, the structures that resembled condensed nuclei or peripherally condensed chromatin seen in HE-stained sections were likely not to be the nuclei of dead tumor cells *per se*, but might be the phagosomes containing nuclear residuals.

Disruption of macrophages and release of cell debris and processed phagosomes into the intercellular space were very commonly observed (fig 4H, 4I, and 4J). On the other hand, formation of cell membrane blebs and apoptotic bodies in dying or dead tumor cells were not observed. Disruption of dead tumor cells was rarely seen. These results again suggest that the cell debris in dead cell islands observed in HE stained sections might be derived mainly from disrupted macrophages.

Expression of cytochrome c, AIF, and CD68

Since the mitochondria in the tumor cells were found to be malformed and frequently degenerated, we performed immunohistochemical staining of cytochrome c and apoptosis-inducing factor (AIF), two mitochondrial proteins known to mediate caspase-dependent and caspase-independent apoptosis, respectively (Loeffler and Kroemer 2000; Skulachev 1998; Daugas *et al.* 2000a, 2000b; Lorenzo *et al.* 1999), although AIF is also related to necrosis (Daugas *et al.* 2000a). Most tumor cells in the major tumor areas and some cells in the dead cell islands exhibited positive staining of cytochrome c in the cytoplasm (fig 5A to 5D). However, to our surprise, the staining intensity was much weaker, compared to the staining in the tumor foci or in the smooth

muscle cells that could function as positive controls (fig 5A and 5B). This result suggests that a higher expression of cytochrome c may be used as an additional marker for the tumor foci. Under high magnification, the staining was found to be granular in the cytoplasm (fig 5C and 5D), indicating that the cytochrome c might still be localized in the mitochondria. Western blot assay of total c-*myc* tumor tissue revealed a single band at the expected molecular weight (fig 6), confirming the specificity of the antibody. A normal rabbit IgG was used to replace the primary antibody in some control staining; this control did not result in any signal (not shown).

Immunohistochemical staining for AIF showed that some, but not all, cells in the dead cell islands had positive nuclei, while some cells in the major tumor areas were negative or very weakly positive in the cytoplasm (but not nuclei) of some cells (fig 5E and 5F). Cells in the foci were negative. Western blot analysis of the total tumor tissue showed a single band at the expected molecular weight (fig 6), confirming that AIF was expressed in the tumor tissue.

Immunohistochemical staining of macrophage marker CD68 gave rise to positive staining in the cytoplasm of many cells. The positive cells were found mainly in the dead cell islands (fig 5G and 5H), usually around the dead cell nuclei, indicating that these nuclei might actually be inside the giant macrophages as phagosomes. The positive cells were also found in the thin stroma among the viable tumor cells (fig H), but were very rare in the foci (not shown). Western blot assay with total tumor tissue lysates showed a single band at the expected molecular weight (fig 6), suggesting the specificity of the antibody. Since the CD68 and AIF antibodies were of goat origin, a normal goat IgG was used to replace the primary antibodies for a control staining; this did not result in any

signal, confirming the staining specificity yielded by the CD68 and AIF antibodies. As both AIF and CD68 antibodies were goat polyclonals, they also functioned as each other's control for the staining specificity.

Discussion

One fundamental question on our observations is whether the formation of a "dead cell island" is a result of the c-*myc* overexpression or is, rather, a common phenomenon for programmed cell death triggered by many factors in a tissue. Although we have no direct evidence to tightly connect c-*myc* overexpression with the formation of dead cell islands, due to limitations in our model system, there are several indications that circumstantially suggest this connection. First, the dead cell islands are observed only in the major tumor areas that show high levels of c-*myc*, but not in the foci that have lost c-*myc* expression, whereas necrosis can occur in both areas (Liao *et al.* 2000a). Second, similar "clusters" of dead cells have also been reported in the polycystic kidney of mice where a c-*myc* transgene was targeted to the renal ducts (Trudel *et al.* 1997). Third, to our knowledge, a similar morphology of dead cell islands has not been described in the literature for cell death triggered by any other factors in any other types of tissue, although this could be due to the lack of detailed morphologic descriptions in most tissues based studies. Nevertheless, the fact that cells in a local neighborhood commit collective suicide suggests that a paracrine mechanism or a cell-cell interaction might be involved in the dying process.

Although cell death in c-*myc* overexpressing tissues has been previously referred to as apoptosis by us (Amundadottir *et al.* 1996; McCormack *et al.* 1998; Liao *et al.* 2000a)

and by other investigators (Hundley *et al.* 1997; Pelengaris *et al.* 2000; Stewart *et al.* 1984; Sandgren *et al.* 1989; Trudel *et al.* 1997; Christensen *et al.* 1999; Sanders and Thorgeirsson 1999), no morphologic evidence other than positive TUNEL staining has been provided to prove that the cells really died of apoptosis in any of these tissues. In this study, we find that cell death in the c-*myc* transgenic mammary tumor is characterized by cell shrinkage, and DNA breakage, as indicated by TUNEL staining, but in the absence of typical apoptotic morphology, i.e. chromatin condensation, cell membrane blebbing, and apoptotic body formation. Moreover, it involves infiltration of a large number of macrophages and their active phagocytosis. As a consequence, it is the macrophages, not the dead tumor cells *per se*, that are frequently disrupted to generate cell debris, and it is their phagosomes, not the apoptotic bodies, that are released to the intercellular space. However, similar to apoptotic bodies, these released phagosomes are processed and membrane-capsulated, and thus do not elicit an inflammatory response. These morphologic features do not seem to fit into the typical description of apoptosis, necrosis, or any other types of cell death provided by the literature, although they are probably closer to "delayed necrosis" or "slow cell death" termed by Blagosklonny (Blagosklonny 2000) to describe cell death without caspase activation. Therefore, the more general term "programmed cell death" is used in our current study to refer the cell death observed. It will be interesting to know if the morphologic properties described herein are specific for the c-*myc* transgenic mammary tumors or if it is common for any of the c-*myc* expressing tissues.

Malformation of mitochondria and depletion of cytochrome c have recently been reported in cultured 3T3 cells that ectopically express Bcl-Xs, an apoptosis-inducing

protein (Fridman *et al.* 2001). In these cells, mitochondria appear less electron-dense, or even transparent. The cristae of mitochondria in these cells are either undeveloped or broken. Similar ulstrastructural malformation of mitochondria is observed in the c-*myc*-induced, c-*myc*-expressing mammary tumor cells in the present study. The malformed mitochondria seem to be fragile, since they are frequently observed to be degenerated. Thus, the present study show for its first time in a tissue that enforced expression of an inducer of programmed cell death may cause malformation and degeneration of mitochondria. Although most tumors survive well with malformed, degenerated mitochondria, it remains possible that the pro-death property of these c-*myc*-expressing cells may be causally related to such mitochondrial alterations. However, since mitochondrial swelling and disruption are marks of early events of necrosis, as distinguishable from apoptosis (Kroemer *et al.* 1998), the cell demise in this model is closer to necrosis than to apoptosis.

Expression of cytochrome c is much lower in dead cell islands and in most tumor cells, compared to the cells in the foci and in the adjacent muscle cells that require high levels of cytochrome c for energy generation. These findings are surprising, and may be explained by either an induction of cytochrome c in the foci or an inhibition in the majority of the tumor cells. No matter which is the case, this is not a general characteristic of mitochondrial proteins, as AIF does not show the same alteration. If the suppression mechanism happens to be the case for cytochrome c, then a question arises as to whether it is related to the high expression of c-Myc, in a way similar to the possible suppression of cyclin D1 by c-Myc shown previously in this tumor tissue (Liao *et al.* 2000a). Also, it is well established that c-*myc*-expressing cells are enhanced for

glycolytic energy generation, due in part to the induction of lactate dehydrogenase gene (LDH-A) by c-*myc* (Osthus *et al.* 2000; Shim *et al.* 1997, 1998a). Alternatively, the higher level of cytochrome c in c-*myc*-silenced tumor foci may also be due to a higher demand of energy generation in association with the increase in the proliferative rate of the foci (Liao *et al.* 2000a). This energy demand may be exacerbated due to the mitochondrial malformation and degeneration in tissue giving rise to the foci.

Glucose depletion has been shown to induce apoptosis of c-*myc*-expressing Rat1 cells in culture (Shim *et al.* 1998b). The aforementioned Bcl-Xs-expressing cells, with mitochondrial malformation and cytochrome c depletion, also undergo caspase-independent cell death (Fridman *et al.* 1999, 2001). The present study shows the similar damage of mitochondria and low level of cytochrome c in the c-*myc* expressing mammary tumor cells that have a high potential for programmed cell death. Putting these data together, one may hypothesize that insufficient generation of energy, which may be caused by damage in mitochondria or depletion in cytochrome c and glucose, may also be capable of inducing programmed cell death. However, since apoptosis is defined as a death process that involves consumption of energy (Blagosklonny 2000), this type of cell demise may not actually be apoptosis by this strict definition. Support for this consideration is the observation that the cell death of the c-*myc* transgenic mammary tumors does not exhibit typical nuclear morphology of apoptosis. Cell death associated with depletion of cytochrome c may be most closely related to the TNFα and the death receptor pathways, but not to the death initiated by common forms of cellular stress such as UV irradiation (Li *et al.* 2000; Vier *et al.* 1999).

In another reported study (McCarthy *et al.* 1997), when Rat1/c-MycER^{TM} cells were induced by c-Myc expression to undergo apoptosis in the absence of caspase inhibitors, they showed typical apoptotic morphology, such as chromatin condensation, cell shrinkage, cytoplasmic blebbing, and cell fragmentation. In contrast, when the cells were induced to undergo apoptosis in the presence of caspase inhibitors, although the cells still showed cytoplasmic blebbing, their nuclei remarkably resembled those of non-apoptotic cells. These results indicate that c-*myc*-triggered cell death may have both caspase-dependent and caspase-independent mechanisms; in the latter situation, the nuclear morphology was similar to what was observed in our study. Localization of AIF (a factor related to the caspase-independent cell death (Lorenzo *et al.* 1999) to the nucleus of the dead cells in the present study seems to suggest that the cell death may be caspase-independent (Daugas *et al.* 2000a, 2000b). The low level of cytochrome c, an important mediator of caspase activation (Skulachev 1998), in the majority of the tumor cells is in line with this possibility and further suggests that the cell demise may not require a high intracellular energy level. Therefore, the lack of nuclear apoptotic features and the immunohistochemical properties of cytochrome c and AIF lead us to the consideration that a caspase-independent pathway may be the mechanism behind the cell death in the c-*myc* transgenic mammary tumors, since it now seems to be clear from the literature that inhibition of caspases can prevent nuclear apoptotic changes (Kolenko *et al.* 1999; Hirsch *et al.* 1997). Thus, the morphologic features observed in the present study might just be another reflection of the differences between caspase-mediated apoptosis and caspase-independent, non-apoptotic programmed cell death, an issue at hot debate in the literature. Whether this is the case requires more thorough studies on the

morphology of the dying cells and on the status of caspase activation for different types of cell death.

In summary, c-*myc* transgenic mammary tumor cells exhibited malformation and degeneration of their mitochondria, with a much lower expression level of cytochrome c than in the c-*myc*-downregulated, progressing foci, for which a higher expression level is an additional marker. Dead cells in this c-*myc* transgenic tumor tissue were large in number and appeared in clusters, a histological feature termed "dead cell islands". The dying cells manifested cell shrinkage, DNA breakage, as indicated by positive TUNEL staining, and nuclear localization of AIF, but without typical apoptotic morphology, i.e. nuclear condensation and formation of cell membrane blebs and apoptotic bodies. In addition, this cell death process attracted a pronounced infiltration of macrophages but without an inflammatory response. Disruption of dead tumor cells was rare, but disruption of macrophages was common. These morphologic features suggest that the cell death process of the c-*myc* transgenic mammary tumor cells may not be typical apoptosis,.but that it may be related to the pre-existent alterations in mitochondria and to the possibly resulting insufficiency in energy generation. These results raise a fundamental question as to whether the cell death appearing in other c-*myc*- expressing tissues is also distinct from classical apoptosis.

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Figure legends

Fig 1. Hematoxylin and eosin (HE) staining of c-*myc* transgenic mammary tumor tissue. A glandular type of focus (**A**) within a c-*myc* tumor does not show any dead cell islands, in contrast to the many such islands in its surrounding tumor area (arrow). However, there are clusters of dying or dead tumor cells (arrowhead) in the cavities of the tumor glands within this type of focus (**A** and **D**). A focus (**B**) shows a mixed morphology of solid and glandular types of tumor cells; an arrow indicates a tumor gland. A tumor area at low magnification (**C**) shows many dead cell islands (arrow). A group of cells (arrow in **E**) are separated from their surrounding tumor cells by an intercellular space that might have resulted from cell shrinkage; at this time point, the morphology of these cells is still similar to the surrounding cells. Cells in dead cell islands showed nuclear fragments (arrow in **F**, **G**, and **H**). There are many small nuclear fragment-like components inside one giant cell (arrowhead in **F**), which is likely to be a macrophage. In a dead cell island, one cell seems to show a condensed nucleus (arrowhead in **G**), while cell debris appears in another dead cell island (arrow in **I**).

Fig 2: Toluidine blue staining of c-*myc* transgenic mammary tumor tissue that was pre-stained with osmium tetroxide and uranyl acetate. Two types of tumor cells, i.e. light and dark cells, were identified (**A** and **B**). Note that few dead tumor cells appear in the dead cell islands (**A** and **B**), except several huge macrophages (arrow) containing many intensely stained phagosomes. Macrophages could also appear among the viable tumor cells (**C**). Some phagosomes show a staining density as light as the cytoplasm of viable

tumor cells, but contain intensely stained components within the phagosomes (arrowhead in **A**, **B** and **D**). These light-stained phagosomes could contain entire engulfed dead tumor cells, while the dark components within them might be nuclear fragments. A disrupted cell is indicated in **E**; its large body size and phagosomes suggest that it may be a dead macrophage.

Fig. 3: TUNEL staining (brown color) of c-*myc* transgenic tumor tissue (**A**, **C**, and **D**) and hyperplastic lesion (**B**), with hematoxylin-counter staining (blue color). Positive nuclei in the tumor tissue appear in clusters to form dead cell islands (**A**, **C** and **D**), whereas positive nuclei in a hyperplastic lesion adjacent to a tumor area appear individually, without formation of dead cell islands. The chromatin density of the positive nuclei in dead cell islands is similar to that of the viable tumor cells. There is no cytoplasm surrounding most of the positive nuclei within dead cell islands, although the cytoplasm of the viable tumor cells can be discerned clearly by its weak hematoxylin staining. There are some small positive particles embedded in cytoplasm (arrow in **C** and **D**), which could be nuclear fragments inside macrophages. Several positive nuclei are encapsulated by cell membrane (arrowhead in **D**), indicating that they might be phagocytosed by a macrophage.

Fig. 4: Ultrastructure of c-*myc* transgenic tumor tissue. According to their electron-density, the tumor cells could be divided into two categories of dark cells and light cells (**A**). Most tumor cells show one or more giant nucleoli (**A** and **B**). Mitochondria (arrow) of the tumor cells show either electron-light (**B** and **C**) or electron-dark (**D**) amorphous

matrix, with absent or very few cristae. When the cristae appear, they may be dilated to cavities with irregular shapes (arrow in **D**). Frequently, the malformed mitochondria are degenerated, characterized by lysis of matrix and cristae, leaving a hollow mitochondrial body (arrow in **E**). Macrophages appear frequently in the tumor tissue, especially in the places of dead cell islands (**F** and **G**). Every macrophage contains many phagosomes (arrowhead in **F** to **J**). Some phagosomes show very high electron density (**H** and **J**) or contain components with very high electron density (**I**). Many phagosomes could be as large as a nucleus and appear in dead cell islands (**G**), and some resemble the nucleus of an engulfed cell (arrow in **F**). Note that in the place of dead cell islands (**G**), tumor cells do not show condensed nuclei. Disruption of the cell membrane and release of phagosomes is seen in a macrophage (arrow in **J**).

Fig 5: Immunohistochemical staining of cytochrome c, AIF and CD68 in c-*myc* transgenic mammary tumor tissue. Staining of cytochrome c is positive in major tumor areas of the tumor tissue and in some (but not all) dead cell islands (arrow in **C**), but the staining intensity is much weaker compared to the smooth muscle cells (upper-area in **A**) and foci (**B**, **C** and **D**). Under high magnification, the staining of cytochrome c in both foci and surrounding tumor area is granular in the cytoplasm (**D**). AIF staining is localized to some nuclei in the dead cell islands (**E** and **F**). Staining for CD68, a marker of macrophages, identifies the positive cells in dead cell islands (**G**), in stroma, and among the tumor cells (**H**). The staining is localized to the cytoplasm and probably also cell membrane, in contrast to the nuclear staining of AIF.

Fig 6: Western blot analysis detects cytochrome c, AIF and CD68 proteins in protein lysates prepared from three (marked 1, 2 and 3) randomly selected c-*myc* transgenic mammary tumors.

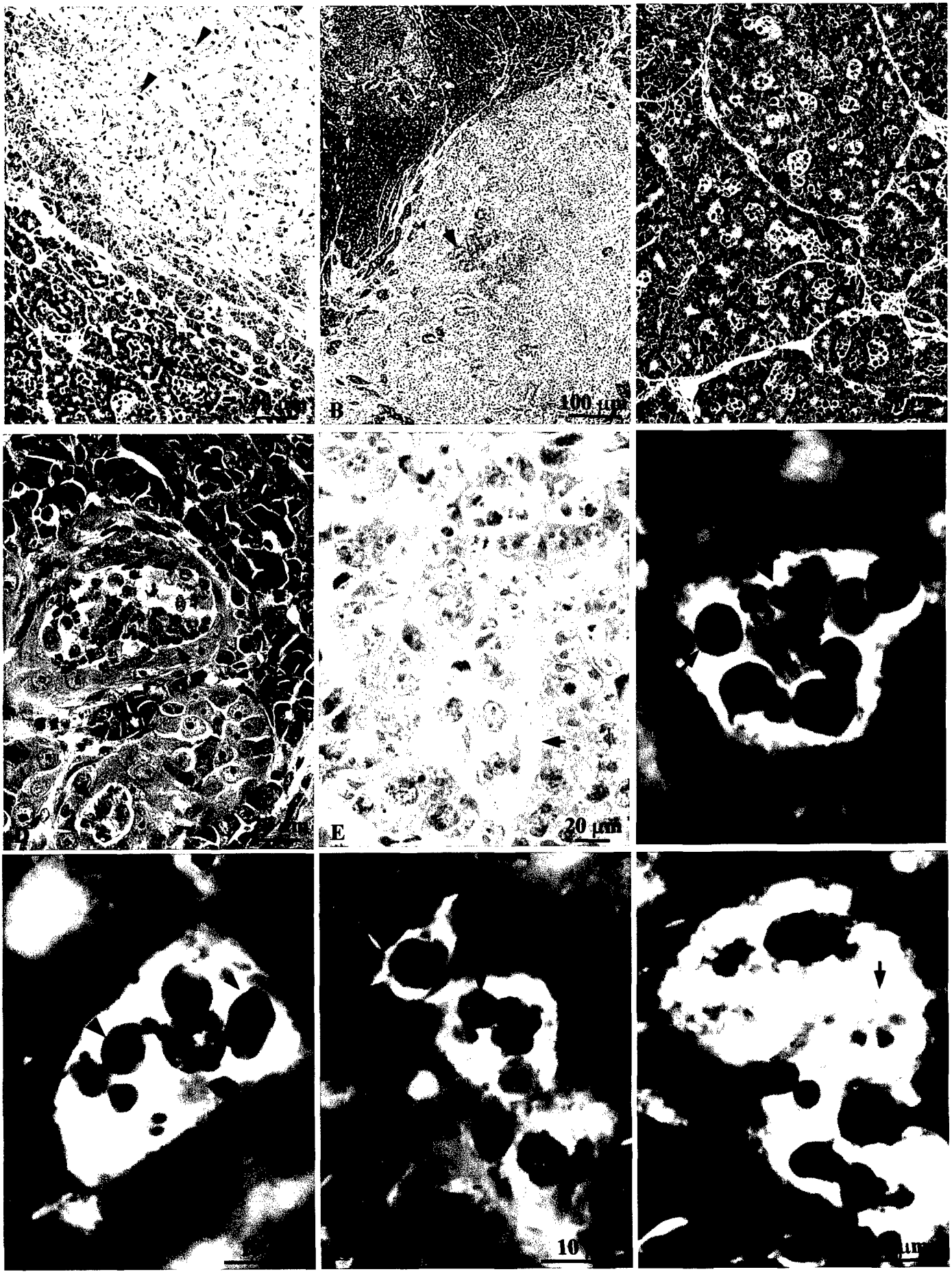

Figure 1

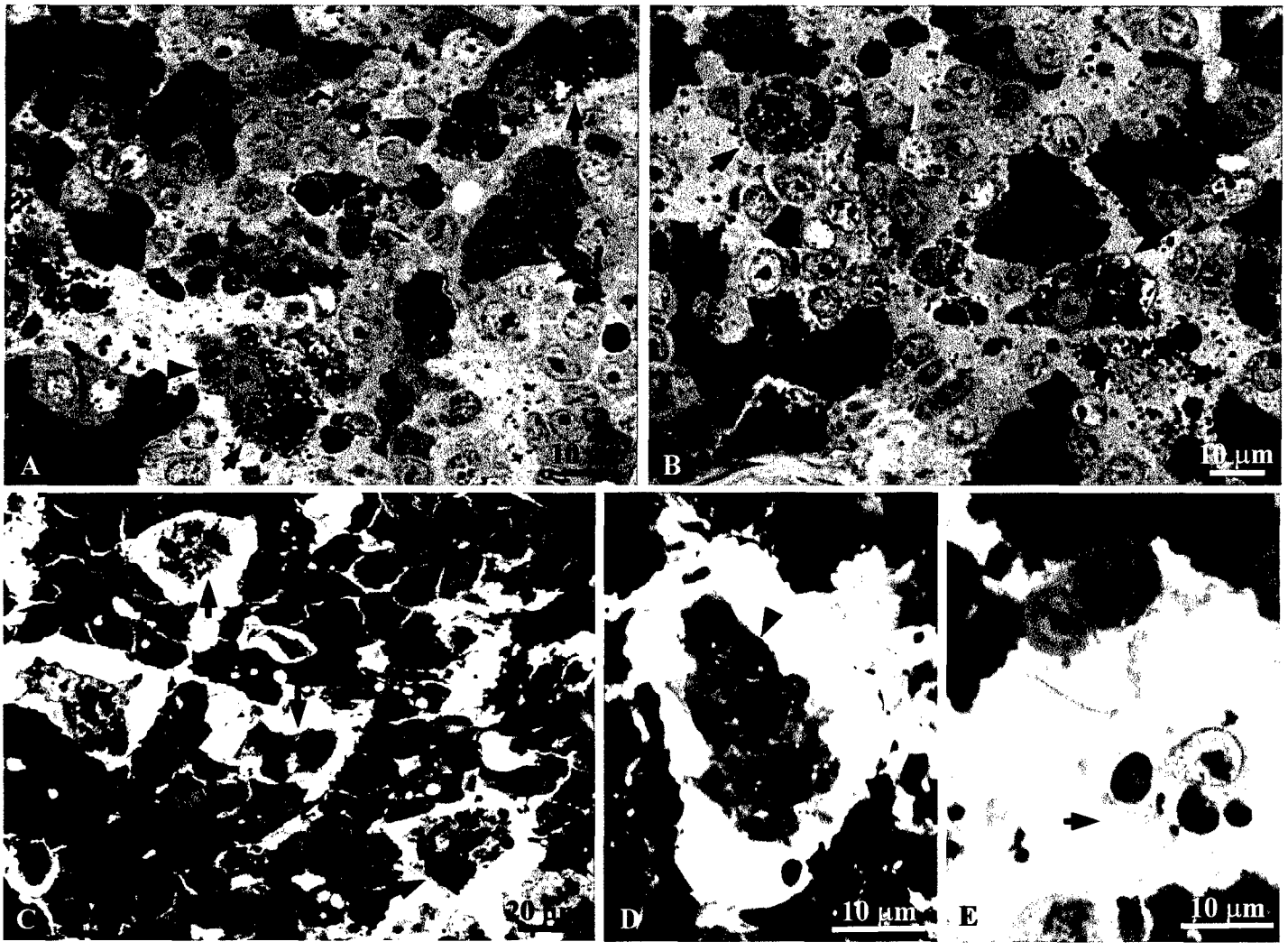

Figure 2

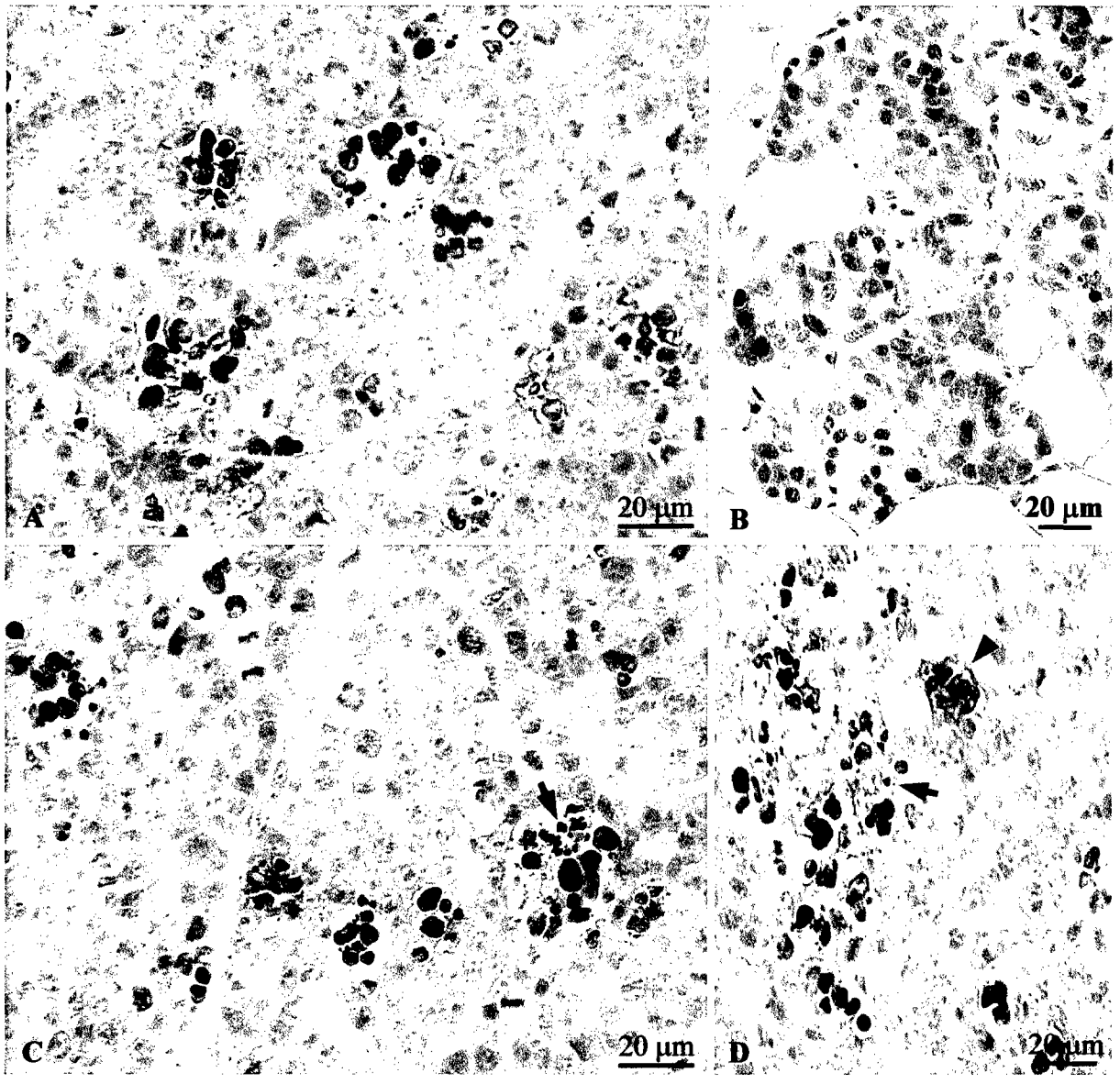

Figure 3

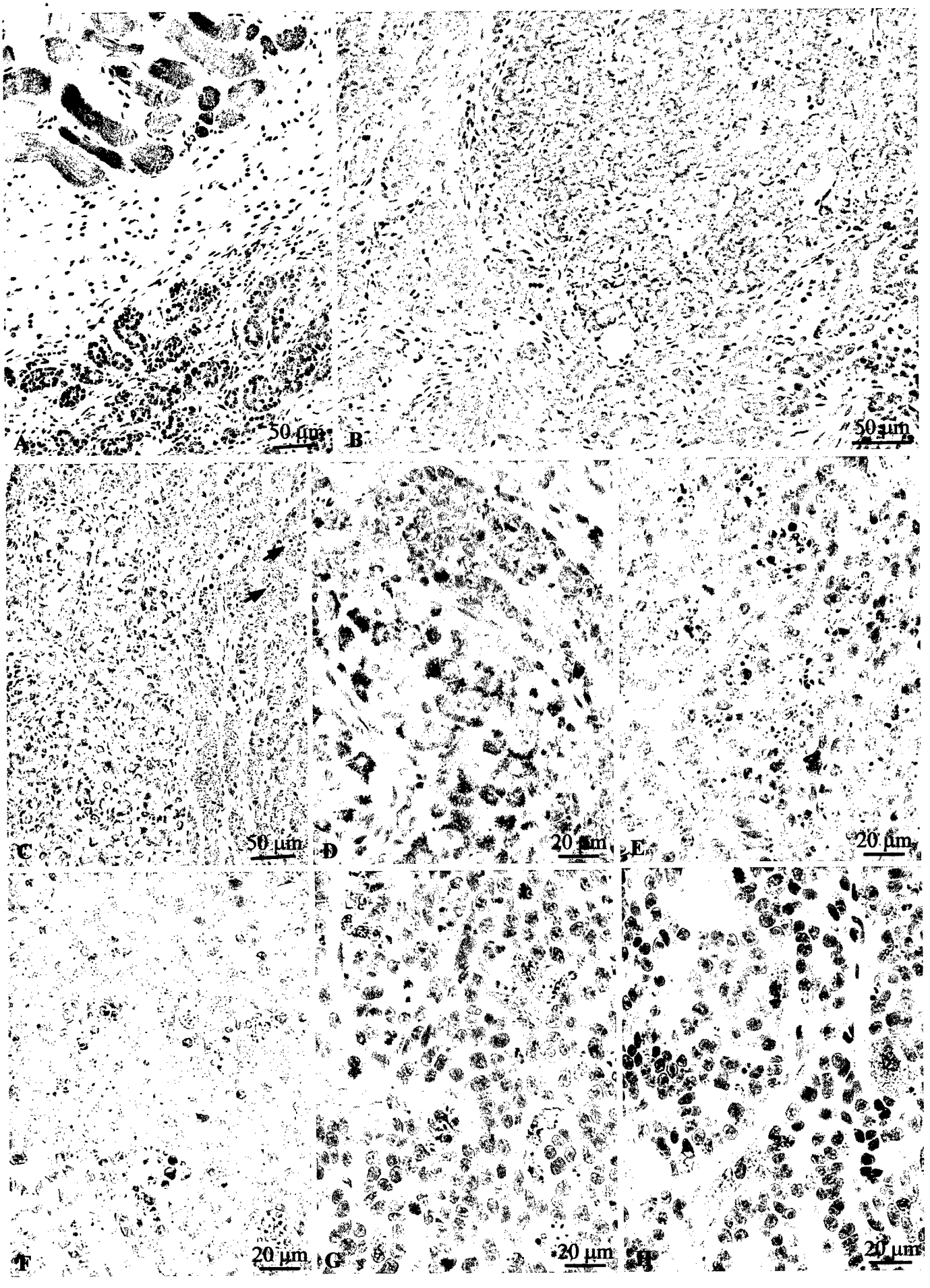

Figure 4

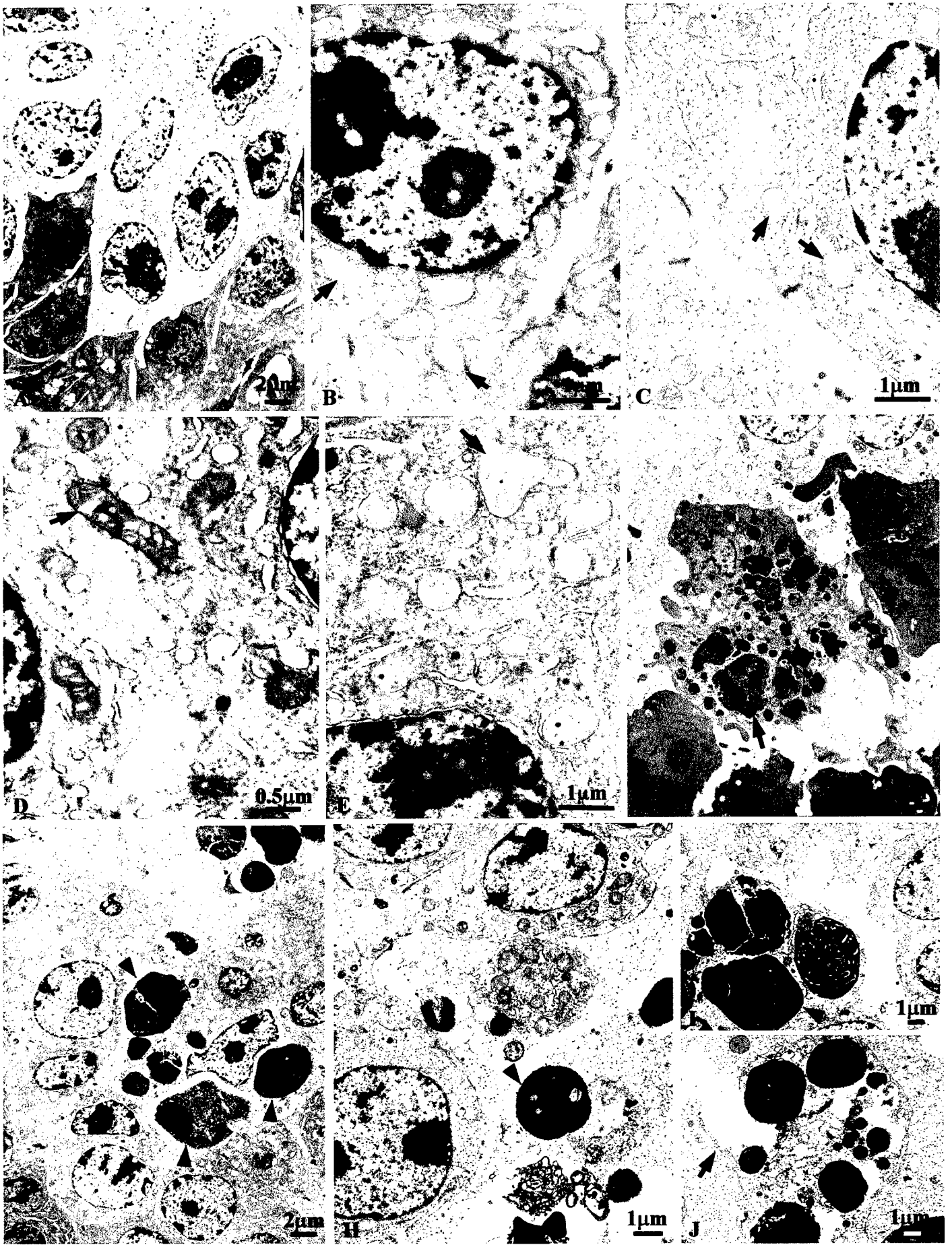

Figure 5

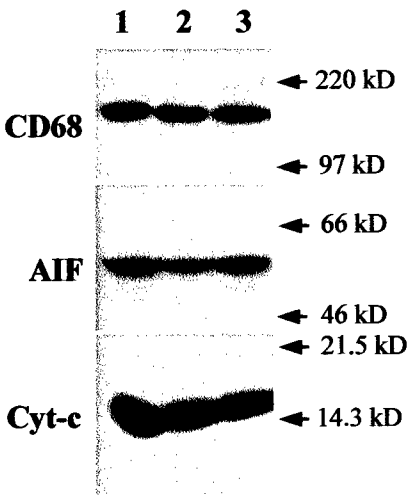

Fig. 6

Appendix IV

Amplification and overexpression of the c-*myc* oncogene in high-grade breast cancer: FISH, *in situ* hybridization, and immunohistochemical analyses

Jan Blancato[1,2], Robert B. Dickson[2,3,4], Xu-fang Pei[2,3], and Dezhong Joshua Liao[*,2,3]

[1]Institute for Molecular and Human Genetics, [2]Lombardi Cancer Center, [3]Department of Oncology, [4]Department of Cell Biology, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington DC 20007, USA

Running title: c-*myc* in breast cancer

Key words: c-*myc*, breast cancer, gene amplification, gene expression

*Correspondence:

Dr. Dezhong Joshua Liao, Research Building, W416, 3970 Reservoir Road, NW, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007

Tel: (202)687-7341 or 687-2277

Fax: (202)687-7505

Email: liaod@gunet.georgetown.edu

Abstract

Although there have been many reports on the amplification and overexpression of the c-*myc* oncogene in human breast cancer, few of such studies have utilized *in situ* hybridization approaches to directly analyze the gene amplification and RNA expression on tumor tissue sections. In this study, we analyzed gene amplification, RNA expression, and protein expression of the c-*myc* gene in tissue specimens of high-grade breast cancer with the techniques of fluorescent *in situ* hybridization (FISH), non-radioactive *in situ* hybridization, and immunohistochemistry. A surprisingly high proportion (70%) of the tumor cases was found to demonstrate amplification of the c-*myc* gene. However, the level of amplification was surprisingly low, ranging between 1-4 copies of gene gains, and the majority (84%) of the cases with the gene amplification gained only 1-2 copies. Approximately 95% and 79% of the cases exhibited c-*myc* RNA and protein overexpression, respectively; in contrast, reduction mammoplasty-derived breast tissue expressed the mRNA and the protein only in about 14% (1 of 7) and 0% (0 of 7) cases, respectively. No statistically significant correlation was identified among the gene amplification indices, the RNA expression scores, and protein expression scores; this result may be related in part to the great intratumoral heterogeneity of c-*myc* expression at both RNA and protein levels. In nearly all of the tumor samples, patches of tumor cells were observed that were both positive and negative for expression of the mRNA and proteins of c-*myc* gene, irrespective of the gene amplification status. No specific tumor histology was identified specifically for the positive expression nor the negative expression of c-*myc* at either RNA or protein level. Some patches of tumor cells that were negative for RNA expression were positive for the protein, and vice versa. Predominantly nuclear staining of c-Myc protein was found in stromal fibroblasts, in hyperplastic and dysplastic lesions, in well-differentiated tumors, as well

as in invasive tumor areas. However, predominantly cytoplasmic staining of c-Myc was observed only in the widely invasive tumor cells, not in fibroblasts, hyperplastic or dysplastic lesions, nor well-differentiated tumor areas. These results indicate that high-grade breast cancer may have high frequency but low copy numbers of the c-*myc* gene amplification, as well as high frequencies of overexpression at the RNA and protein levels. The gene amplification, RNA overexpression and protein overexpression of c-*myc* are not necessarily interrelated. The occurrence of predominantly cytoplasmic localization of c-Myc protein may correlate with the tumor aggressiveness and thus a poor prognosis.

Introduction

The c-*myc* oncogene has been shown amplified and/or overexpressed in many types of human cancer (1-4). Numerous *in vivo* experiments have also causally linked aberrant expression of this gene to the development and progression of cancer at different body sites (1-4). However, several critical issues regarding the significance of c-Myc to human cancer still remain obscure. First, even for a given type of malignancy, the frequencies of the alterations of c-*myc* at the cytogenetic and expression levels vary greatly from one report to another (3). For instance, the frequencies for its amplification, RNA overexpression and protein overexpression in breast cancer vary between 1-94%, 22-95%, and roughly 50-100%, respectively, among different clinical reports (3). Thus, it is still unclear to what extent this gene is altered at the cytogenetic level and at different expression levels in cancers of the breast and other body sites.

Another controversial issue pertains to the prognostic value of c-Myc alterations in cancer. The central role of c-Myc in accelerating cell proliferation documented by many early studies has led to a general concept for many types of cancer that amplification or overexpression of this gene may be associated with a more aggressive tumor and a poorer patient survival (1-7). However, many reports have also shown the opposite correlation (8-19), while other studies do not support either of these conclusions. For instance, amplification or overexpression of c-*myc* has also been shown to associate with a better tumor differentiation or a better patient survival for the cancer of the testis, ovary, bile ducts, colon, and breast (8-19). This controversy may not completely be related to the cancer type, since both positive (5-7) and negative (8-11,18,19) correlations have been reported for colon cancer and breast cancer. More interestingly, c-Myc overexpression has been shown to predict a poorer prognosis for cutaneous melanoma but a better outcome for uveal melanoma (20-24), although different opinion still exists (25). These

data indicate different roles of c-Myc, even in the same type of tumor, depending upon different microenvironments.

A third unsolved issue concerns the intracellular localization of the c-Myc oncoprotein. c-Myc functions as a transcription factor and therefore should be localized to the nucleus. However, in many immunohistochemical studies with different c-Myc primary antibodies, the c-Myc protein is observed not only in the nucleus but also in the cytoplasm or in both nucleus and cytoplasm (25-28). Some studies showed that the cytoplasmic immunostaining might be an artifact of formalin-fixation of the tissue (29,30), whereas other experiments showed that it was irrelevant to the fixation (31). Nevertheless, recent western blot analysis with nuclear extracts and cytoplasmic extracts have demonstrated the existence of c-Myc protein in both nucleus and cytoplasm (27). Some reports have revealed that it is the cytoplasmic immunostaining, but not the nuclear staining, that is associated with the tumor grade, tumor differentiation, tumor invasion, and/or patient survival (32-35). Furthermore, studies on neoplasm of the colon, testis, ovary, and liver have shown that predominantly nuclear localization of c-Myc tends to occur in benign lesions, while predominantly cytoplasmic localization tends to occur in more malignant tumors (11,13,32,36,37). Whether these patterns of subcellular localization of c-Myc tend to reflect the malignant status of breast cancer remains an open question.

To begin to address more clearly these issues in breast cancer, we studied the amplification and overexpression of the c-*myc* gene with fluorescent *in situ* hybridization (FISH), non-radioactive *in situ* hybridization, and immunohistochemical approaches on paraffin-embedded biopsy sections of untreated high-grade breast cancer. It was observed that 70%, 95%, and 79% of the cancer cases exhibited c-*myc* gene amplification, RNA overexpression, and protein overexpression, respectively. In most (84%) cases that showed the gene amplification, the c-*myc* gene gained only 1 to 2 copies. Nuclear localization of the c-Myc protein occurred in normal,

benign, or malignant cells, whereas cytoplasmic localization, with or without additional nuclear localization, occurred only in those invasive tumor cells. Thus, cytoplasmic localization of c-Myc may correlate with the tumor aggressiveness and possibly also poor prognosis.

Materials and methods

Materials. Formalin-fixed, paraffin-embedded tissue blocks of human breast cancer and normal breast tissue were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Cancer Center at Georgetown University Medical Center. The criteria for tumor selection were the following parameters: negative progesterone receptor status, positive lymph node involvement, and high tumor grade. The parameters were chosen from our prior meta analysis (38) as indications of a high likelihood of c-*myc* amplification. Normal breast tissue specimens were from reduction mammoplasty. Serial sections (5 μm) of individual tissue blocks were prepared by the Histopathology Laboratory for the analyses of FISH, *in situ* hybridization, and immunohistochemistry.

FISH. A dual-label FISH technique was used (39). Sections were deparaffinized with xylene and re-hydrated with a graded series of 70%, 80%, and 95% ethanol at room temperature. Samples were then digested with 4% pepsin (Sigma, St. Louis, MO) at 45 °C for 10 minutes. DNA probes used were an alpha satellite probe to chromosome 8, labeled with biotin, and a *c-myc* probe labeled with digoxigenin (Ventana, Tucson, AZ). Detection of signals was accomplished with an anti-avidin antibody labeled with Texas Red, and an anti-digoxigenin antibody conjugated to fluorescein (Ventana, Tucson, AZ). Slides were post-washed in 2xSSC at 72 ^{0}C for 5 minutes and counterstained with DAPI to visualize cell nuclei. Results were viewed and quantified with a Zeiss Axiophot fluorescence microscope equipped with appropriate filters and an Applied Imaging Cytovision system (Pittsburgh, PA). In this approach, the c-*myc* unique

sequence probe was visualized as a green signal and the control probe for the chromosome 8 centromere was red, thus easily being distinguished when scored.

One serial section from each tumor sample was stained with hematoxylin and eosin and first reviewed by a pathologist, to help identify the tumor area of the section. This procedure ensured that the tumor cells, but not the normal cells, were counted. Nuclei of 20 tumor cells were scored from each FISH-stained section independently by two investigators. Hybridization signals were averaged and the amplification index was presented as the number of *c-myc* signals divided by the number of chromosome 8 centromere signals. A 1.8-fold increase was used as the criterion to judge the presence of gene amplification, as used by other investigators (40).

***In situ* hybridization.** *In situ* hybridization was carried out with a non-radioactive method described previously (41,42). One serial section from each specimen was hybridized overnight at 60 °C with riboprobes that were *in vitro* transcribed from the antisense or sense strand of an approximately 300bp cDNA of human c-*myc* (EST11414L, ATCC, Manassas, VA), labeled with digoxigenin-conjugated UTP. The sections were then incubated with an antibody against digoxigenin, followed by incubation with a second antibody conjugated to alkaline phosphatase. The signal was visualized by color development with 5 bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All reagents were purchased from Boehringer Mannheim, Indianapolis, Indiana. To control the signal specificity, two serial sections were mounted on the same slide for hybridization with the antisense and sense probes, respectively. The cut-off for positive cases for the mRNA expression was subjectively set at 10% or more of tumor cells that showed clearly stronger staining than the cells hybridized with the sense probe. The expression levels were subjectively scored as –, +, ++, +++, in order of the negative to the strongest positive.

Immunohistochemistry. Immunohistochemical staining was performed using an avidin-biotin complex (ABC) method described previously (43). One serial section of each specimen

was deparaffinized and blocked with 3% peroxide. Antigens were retrieved by heating in a microwave oven in 50 mM citrate buffer, pH 6.0, after boiling for 12 minutes. After blocking with 6% normal goat serum, the section was incubated with a mouse monoclonal antibody to human c-Myc (9E10, Sigma Chemical Company, St. Louis, MO) for 2 hours, followed by 1 hour incubation with a second antibody conjugated with biotin (Vector Laboratories Inc., Burlingame, CA). The section was then incubated with peroxidase-conjugated avidin (Dako, Corporation, Carpinteria, CA) for 30 minutes, followed by color development with diaminobenzidine and peroxide. All procedures were carried out at room temperature. To control the signal specificity, serial sections from ten tumor samples were also stained by using another c-Myc antibody (C19 from Santa Cruz Biotechnology Inc., Santa Cruz, CA). This antibody resulted in positive staining in the same patches of tumor cells as seen in the staining by using 9E10 antibody, but the staining intensity was weaker. Also to control the signal specificity, serial sections were made from five selected positive cases and were subjected to the same staining procedure, with a normal mouse IgG to replace the c-Myc antibody. This control staining did not give rise to a signal, demonstrating the specificity of the signal given by the c-Myc antibody. The cut-off for positive cases was subjectively set at 10% or more of tumor cells that showed clear staining. The expression levels were subjectively scored as -, +, ++, +++, in order of the negative to the strongest positive.

Statistical Analyses. For each analysis of gene amplification, RNA expression, and protein expression, all cases were first grouped as positive or negative to calculate the percentages of positive cases and negative cases. The χ^2 test was used for the comparisons between the percentage of the cases with gene amplification and the percentage of the RNA positive cases, between the percentage of the cases with gene amplification and the percentage of the protein positive cases, as well as between the percentage of mRNA positive cases and the percentage of

protein positive cases. To further estimate the association of the amplification indices with the RNA expression levels, the 4-categories of RNA expression levels (-, +, ++, +++) were first converted to the scores of 1, 2, 3, and 4, respectively. Each amplification index was paired with its corresponding RNA expression score to calculate the coefficient *r*. The same method was used to estimate the association of the amplification indices with the c-Myc protein expression levels, and the association of the RNA expression levels with the protein expression levels. A *p* value of 0.05 was used to determine the statistical significance in all analyses.

Results

FISH analysis of gene amplification

Amplification of the c-*myc* gene was measured by a FISH test in 46 cases of breast cancer; a representative photograph of these results is shown in Figure 1. A 1.8-fold increase cut-off was calculated by the number of *c-myc* signals divided by the number of chromosome 8 alpha satellite signals, and was used to judge gene amplification. As shown in Table 1, 32 of 46 (70%) cases were gene amplified for c-*myc*, whereas only 30% (14/46) of the cases showed amplification indices lower than the cut-off value. However, the amplification indices for most (84%, or 27/32) cases with gene amplification ranged between 1.8 and 3, indicating that the locus gained only 1 to 2 copies of c-*myc* in the majority of the cases. The percentage of cases with gene gains of 3 copies or higher was only 11% (5/46) of total cases analyzed or near 16% (5/32) of the cases with gene amplification, including 1 case (2% of total cases or 3% of the cases with gene amplification) with the highest index of 5 (a gain of 4 copies). The FISH technique required a fluorescence microscopic observation, and therefore the topographic relationship between gene amplification and tumor morphology was not studied.

In situ hybridization analysis of c-*myc* mRNA expression

A total of 62 breast cancer samples, including those subjected to the FISH analysis, were studied for c-*myc* mRNA expression, using *in situ* hybridization with a non-radioactive technique. Hybridization with an antisense probe localized the mRNA to the cytoplasm of the positive cells (Fig 2a), in strong contrast to the weak, background staining in the whole cells on the sections hybridized with the sense probe included as controls (Fig 2b).

As shown in Table 2, 95% (59/62) of the tumor cases were positive RNA expression, slightly but significantly higher than the percentage of the cases with gene amplification ($p<0.05$). Of the 3 cases negative for the mRNA expression, 2 were positive, but 1 was negative, for gene amplification. The mRNA positive cases were distributed, without significant difference, among those with and without the gene amplification. Correlation analysis did not identify any significant association between the RNA expression levels and the gene amplification indices. In addition, 1 of 7 normal mammary tissue samples obtained from reduction mammoplasty unexpectedly exhibited moderate expression of c-*myc* mRNA in about 50% of the mammary gland epithelial cells.

In tumor specimens, the positive staining was localized mostly to the tumor cells (fig 2a), sometimes to the fibroblasts and the endothelial cells of blood capillaries and small veins in the tumor stroma. Even when the tumor cells were negative for the RNA expression, some fibroblasts in the tumor stroma still showed strong positive for c-*myc* expression (Fig 2d). Epithelia in normal mammary glands adjacent to the tumor areas were usually negative for expression of c-*myc*; however, myoepithelial cells surrounding the mammary epithelia often showed positive (Fig 2a). The staining in hyperplastic and dysplastic lesions, which were often adjacent to tumor areas, was variable between negative and moderately positive, but usually weaker than the staining in tumor cells (Fig 2c).

RNA expression usually was heterogeneous in the tumor tissue. Commonly, within an individual tumor, some patches of tumor cells showed strong expression while other patches showed little expression. Even in those cases that were classified to the negative category, there still were some tumor cells individually scoring positive for RNA expression. The morphology of the positive and the negative patches were either similar or very different (Fig 2e, 2f and 2g); no particular tumor morphology was identified specifically for positive or negative expression. However, widely invasive tumor cells, which were usually poorly-differentiated and disseminated in the stromal or adipose tissue, always showed strong expression (Fig 2h and 2i).

Immunohistochemical staining of c-Myc proteins

Fifty-two breast cancer cases, which included those subjected to FISH analysis and had also been analyzed for c-*myc* mRNA by *in situ* hybridization, were also analyzed for the expression of c-Myc protein using immunohistochemical staining with the 9E10 antibody. As shown in Table 2, 79% (41/52) of the cases were positive for c-Myc protein; this was similar to the percentage of the cases for the gene amplification but was significantly ($p< 0.05$) lower than the percentage of the cases positive for the RNA expression. Of the 11 (17%) cases that were negative for the c-Myc protein, 2 were negative, but 9 were positive, for the RNA expression. No significant correlation was identified between the RNA expression levels and the protein expression levels. Of the protein positive cases, 81% were positive, whereas 19% were negative, for gene amplification. Of the protein negative cases, 69% were positive, whereas 31% were negative, for gene amplification. Statistical analysis did not identify any correlation between the gene amplification indices and the protein expression levels. All 7 specimens of normal mammary tissue from reduction mammoplasty exhibited negative staining for c-Myc protein.

Expression of c-Myc protein was mainly localized to tumor cells or to the cells in the hyperplastic and dysplastic lesions (Fig 3a and 3b), although fibroblasts in tumor stroma sometimes were also positive. Epithelia in the normal mammary glands near the tumor areas were usually negative; however, as seen for the RNA expression, myoepithelial cells surrounding the normal epithelia were usually positive in the nucleus (not shown). Similar to RNA expression, in almost every tumor, there were patches of tumor cells showing strong staining, while other patches exhibited negative expression. Some patches that were negative for the RNA expression were still positive for the protein, and vice versa. In the surrounding of negative patches, there often were some fibroblasts showing moderately positive staining. Even in those 11 negative samples, many fibroblasts (Fig 3c) and a small number of tumor cells still exhibited moderate staining. Therefore, the negative staining of tumors is unlikely to be an artifact of tissue fixation or processing.

The c-Myc protein could be localized to the cytoplasm, nucleus, or both. Hyperplastic and dysplastic lesions mainly showed nuclear staining (Fig 3b), while relatively better-differentiated tumor areas exhibited predominantly nuclear staining with weak cytoplasmic staining in some cells (Fig 3d). On the other hand, poorly-differentiated, widely invasive tumor cells that were disseminated in the stroma or in the adipose tissue showed any of these three patterns: predominantly nuclear staining (Fig 3e), predominantly cytoplasmic staining (Fig 3f), or mixed nuclear and cytoplasmic staining (Fig 3g). The tumor cells that manifested predominantly cytoplasmic staining were usually large in cell sizes and abundant with cytoplasm (Fig 3f). In strong contrast to the nuclear staining, predominantly cytoplasmic staining was never observed in fibroblasts, in hyperplastic lesions, nor in dysplastic lesions. Predominant cytoplasmic staining was seen only rarely in the relatively better-differentiated tumor areas.

To verify the staining specificity, serial sections from ten tumor specimens that were positive for 9E10 antibody were also stained by using the C19 rabbit polyclonal c-Myc antibody. This antibody resulted in the same staining pattern as 9E10. As shown in Fig 3h and 3i, the patches of tumor cells positive for 9E10 were also positive for C19, and vise versa. However, it was common that the staining intensity resulted from C19 was weaker than 9E10. The specificity of these two antibodies had also been verified by using western blot approach in our previous studies (42, 44).

Discussion

Although there have been many reports on c-*myc* amplification in human breast cancer (3), to our knowledge there are only two reports involving application of the FISH technique to unfixed, frozen sections (45,46) and one study using the FISH on tissue microarray (47). Using the FISH technique on formalin-fixed, paraffin-embedded sections, we now show that 70% of high-grade breast cancer samples bear the c-*myc* gene amplification. However, the level of amplification was surprisingly low, ranging between 1-4 additional copies of the gene; the majority (84%) of the cases with the gene amplification gained only 1-2 copies, similar to the data reported for metastatic prostate carcinomas (39). The percentage of the amplified tumors reported herein is much higher than the average figure (15.5%) reported in the literature, independent of tumor grade, as quantified in a recent meta-analysis (38). This could be the result of the much higher sensitivity and precision of the FISH method. In addition, the 70% of amplified tumors in this study are also much higher than the 12% reported by Schraml *et al* using the FISH on tissue microarray (47). This large difference may be because the arrays are further prepared from paraffin-embedded tissue and/or because the arrayed tissues are as small as 0.6 mm in diameter (so-called "micro") and contain too few tumor cells for the analysis. We also consider it likely

that the percentage of the amplified tumors and the copy number of gene gains in low-grade breast cancer be lower than the 70% and the 1-2 additional copies, respectively, noted for the high grade tumors in this study.

Most reports on the expression of c-*myc* mRNA utilized Northern blot, dot blot, or PCR-based approaches while just a few involved *in situ* hybridization, mainly performed on frozen sections (3). Normal breast tissue is dominated by adipose cells, differing greatly from tumor tissue in epithelial cellularity. Thus, normal and tumor tissues may not be rigorously compared by techniques involving RNA extraction from total tissue. Therefore, theoretically, no conclusion like "increased expression" should be drawn from the studies with Northern blot, dot blot and PCR-based techniques that require RNA extraction. Using a more sensitive, non-radioactive *in situ* hybridization approach on formalin-fixed, paraffin-embedded sections, we reported herein that about 95% of high-grade breast cancer biopsies show c-*myc* overexpression, compared either to the normal mammary glands distant from the tumor areas in the same specimen or to the mammary glands from reduction mammoplasty. This figure is much higher than the recently reported data (22%) obtained by using a real-time RT-PCR method (19). Dilution of the RNA from epithelium by the RNA from adipose in normal breast tissue in this latest report may be one of the possible explanations for this large difference.

Although 70%, 95%, and 79% of the tumor samples in our study manifest gene amplification, RNA overexpression, and protein overexpression, respectively, no significant correlation was identified between the indices of gene amplification and the expression at either RNA level or protein levels. One of the possible explanations is that in most cases, overexpression of c-*myc* at the RNA or protein level may involve mechanisms other than gene amplification, such as increased transcription and increased stability of RNA and protein that are well documented in the literature (1-7). The eleven breast cancer specimens that are negative for

the protein but positive for the RNA indicate that the mRNA induction may not necessarily lead to an increase in the protein level. Suppression at different post-transcriptional levels could be involved, which further contributes to the lack of the correlation between the RNA levels and the protein levels. Moreover, great intratumoral heterogeneity in c-*myc* expression at both RNA and protein levels, manifested as positive and negative patches, also makes the statistical comparisons somewhat problematic.

Many studies involving different c-Myc primary antibodies in approaches of immunohistochemistry (11,13,14,25-28,32-36) and western blot (27) have shown a cytoplasmic localization of c-Myc protein, with or without nuclear localization. In the normal mucosa of colon, exclusively cytoplasmic staining was observed in the mature zone, whereas mixed nuclear and cytoplasmic staining was discerned in the maturation zone, with exclusively nuclear staining in the proliferative zone (36). In testis, cytoplasmic staining was observed in normal interstitial and Leydig cells, with predominantly nuclear staining in benign seminomas, whereas mixed cytoplasmic and nuclear staining was discerned in undifferentiated, clinically aggressive malignant teratomas, which was somewhat controversial to the above observations (13). More confusingly, cytoplasmic staining has been shown to associate with the tumor grade, differentiation, invasion, and/or patient survival at least for cancers of ovary, kidney, colon, and breast (32-35). For neoplasm of the colon and ovaries, nuclear localization of c-Myc tends to be more frequent in benign lesions whereas cytoplasmic localization tends to be in more malignant tumors (11,13,32,36,37). Thus, although these confusing data suggest that the subcelullar localization of c-Myc may be interrelated to the status of proliferation or differentiation of a tissue, the picture of such interrelationship is still obscure.

High-grade tumors are commonly very heterogeneous, containing not only poorly-differentiated areas but usually also some well-differentiated areas and even some normal tissue

and benign lesions. We use this heterogeneity to explore the interrelationship between the subcellular localization of c-Myc and the proliferation or differentiation of a tissue, in a topographic manner within each tumor individuals, thus potentially ruling out the possible artifact of tissue fixation and processing. We find that nuclear staining occurs in all normal, benign, well-differentiated, and invasive tumor tissues, which is conflicting with the observation that nuclear c-Myc tends to be more frequent in benign lesions in ovary and colon (11,32,36). In addition, we also find that cytoplasmic staining of c-Myc occurs predominantly in invasive tumor cells and occasionally in better-differentiated tumors, but not in normal cells and benign lesions. These results are consistent with the reports that cytoplasmic c-Myc tends to occur in more malignant tumors in some organs (32-35), but conflicting with the observations that cytoplasmic c-Myc tends to occur in normal, differentiated cells in testis and colon (13,29,36). Thus, it is possible that the interrelationship between the subcellular localization of c-Myc and the status of proliferation/differentiation may be tissue-specific. For the breast cancer, appearance of cytoplasmic staining of c-Myc may correlate with the tumor aggressiveness and possibly a poor prognosis.

In a physiological situation, the c-Myc protein has a very short half-life and should increase only transiently at the G0/G1 transit of the cell cycle. A quick down-regulation after the cell enters G1 phase may be needed for the expression of G1 phase genes, such as cyclins D and E. This may be the reason for the *in vitro* observation that high level of c-Myc suppresses cyclin D1 expression (48,49), and for our recent finding that c-Myc and cyclins D1 and E are reciprocally expressed in different areas of the mammary tumors developed in c-*myc* transgenic mice (42). It is thus possible that normal, benign, and less malignant cells may retain the normal mechanisms for quickly down-regulating the c-Myc level after G0/G1 transit, whereas highly malignant tumors like high-grade breast cancer may have lost such mechanisms while c-Myc expression is

exceptionally high. Therefore, prevention of its entry into the nucleus such as by binding of cdr2 protein to c-Myc (27), resulting in the cytoplasmic accumulation of c-Myc, may be used by the tumor cells as a mechanism to facilitate the expression of G1 phase genes, and thus also cell cycle progression. Further studies on the interrelationship between c-Myc and G1 phase genes are needed to test this hypothesis.

In conclusion, the present study shows that about 70%, 95%, and 79% of biopsies of untreated high-grade breast cancer exhibit c-*myc* gene amplification, RNA overexpression, and protein overexpression, respectively. In most (84%) cases with gene amplification, the c-*myc* gene gains only 1 to 2 copies. Nuclear localization of c-Myc proteins occurs in normal, benign, or malignant cells, whereas cytoplasmic localization, with or without nuclear staining, occurs mainly in those invasive tumor cells, and may thus indicate the tumor aggressiveness and probably also a poor prognosis.

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Figure legends

Figure 1. FISH staining showing c-*myc* amplification in a tumor area. FISH probe of a human c-*myc* unique-sequence was shown as green fluorescence, while alpha satellite probe to chromosome 8 used as the control was shown in red. The nuclei of tumor cells were visualized by counter-staining with DAPI.

Figure 2. Non-radioactive *in situ* hybridization of various breast cancer samples with antisense (***a*** and ***c*** to ***i***) or sense probes (***b***). ***a***: c-*myc* mRNA expression (black staining) was very high in tumor cells (arrow head) but not in the normal epithelial cells adjacent to the tumor area. However, myoepithelial cells surrounding the normal epithelia (arrow) were strongly positive in the cytoplasm. ***b***: Hybridization with a sense probe gave rise to weak background staining in a tumor tissue. A serial section of this tumor mounted on the same slide and hybridized with antisense probe was strongly positive (not shown). ***c***: A hyperplastic lesion (low-left corner) adjacent to the tumor areas (up-right corner) were variable in the staining, from negative (arrow) to moderate positive, but usually weaker than the staining in the tumor cells. ***d***: Fibroblasts in the stroma of an RNA negative tumor strongly expressed c-*myc*. ***e***, ***f***, and ***g***: Patches of tumor cells from 3 individual tumors show either positive or negative (arrows) c-*myc* expression. Note that the morphology of the negative patches (arrows) in these three photos is different from one to another and from the positive patches. ***h*** and ***i***: Widely invasive tumor cells strongly expressed c-*myc*.

Figure 3: Immunohistochemical staining (brown color) of various breast cancer samples with the 9E10 (***a*** to ***h***) or C19 c-Myc antibody (***i***). ***a***: A high-grade breast tumor shows predominantly

nuclear staining of c-Myc. ***b***: c-Myc is localized preferentially to the nuclei of the cells in a hyperplastic lesion. ***c***: In a c-Myc negative tumor, fibroblasts in the stroma still show weak or moderate staining for c-Myc. ***d***: An area of relatively better-differentiated tumor cells showed predominant nuclear staining. ***e***: Tumor cells invading into stroma show strong nuclear staining. ***f***: Tumor cells invading into stroma exhibit predominant cytoplasmic staining. Note that most tumor cells are much larger in sizes compared to the tumor cells in other photos. ***g***: Many tumor cells show mixed nuclear and cytoplasmic staining while some other tumor cells and some fibroblasts show only nuclear staining. ***h*** and ***i***: The same patch of tumor cells stained by using 9E10 (***h***) or C19 antibody (***i***) shows similar staining in both cytoplasm and nucleus.

Table 1. c-*myc* amplification in high grade breast cancer

Amplification Index	Percentage of samples
<1.8	30.4% (14/46)
>1.8	58.7% (27/46)
>3.0	8.6% (4/46)
>5.0	2.2% (1/46)

*: Index = number of c-*myc* signals / number of control signals. Normal control ratio is 1.

Table 2. RNA and protein levels of c-*myc* in high grade breast cancer

	Expression levels			
	-	+	++	+++
mRNA (62 cases)[a]	**4.8%**	**4.8%**	**46.8%**	**43.5%**
Proteins (52 cases)[b]	**21.2%**	**21.2%**	**36.5%**	**19.2%**

a: mRNA level is scored by *in situ* hybridization.

b: Protein level is scored by immunohistochemical staining.

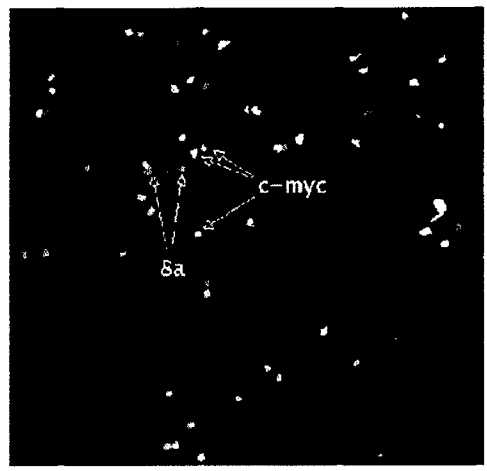

Fig. 1

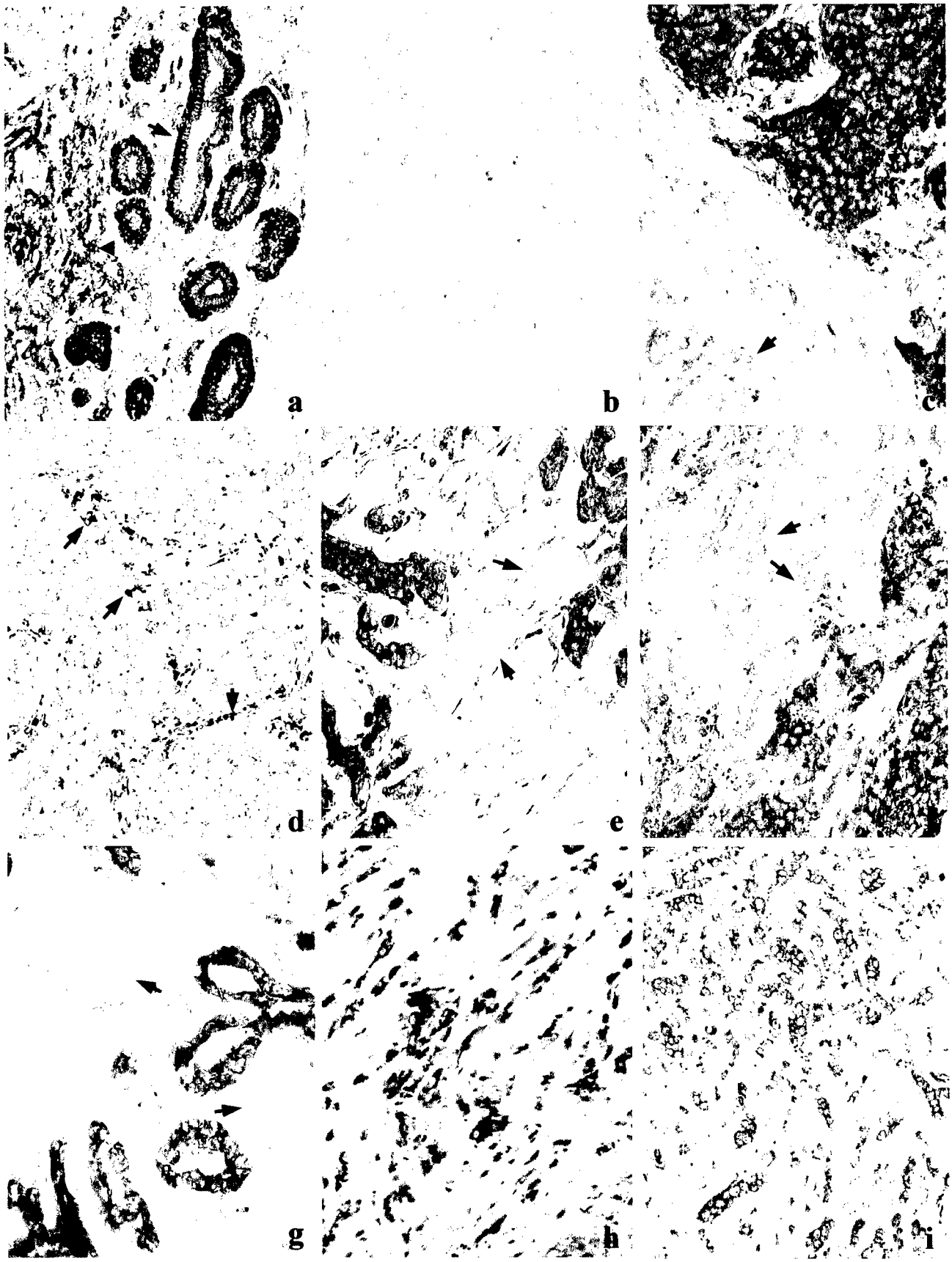

Figure 2

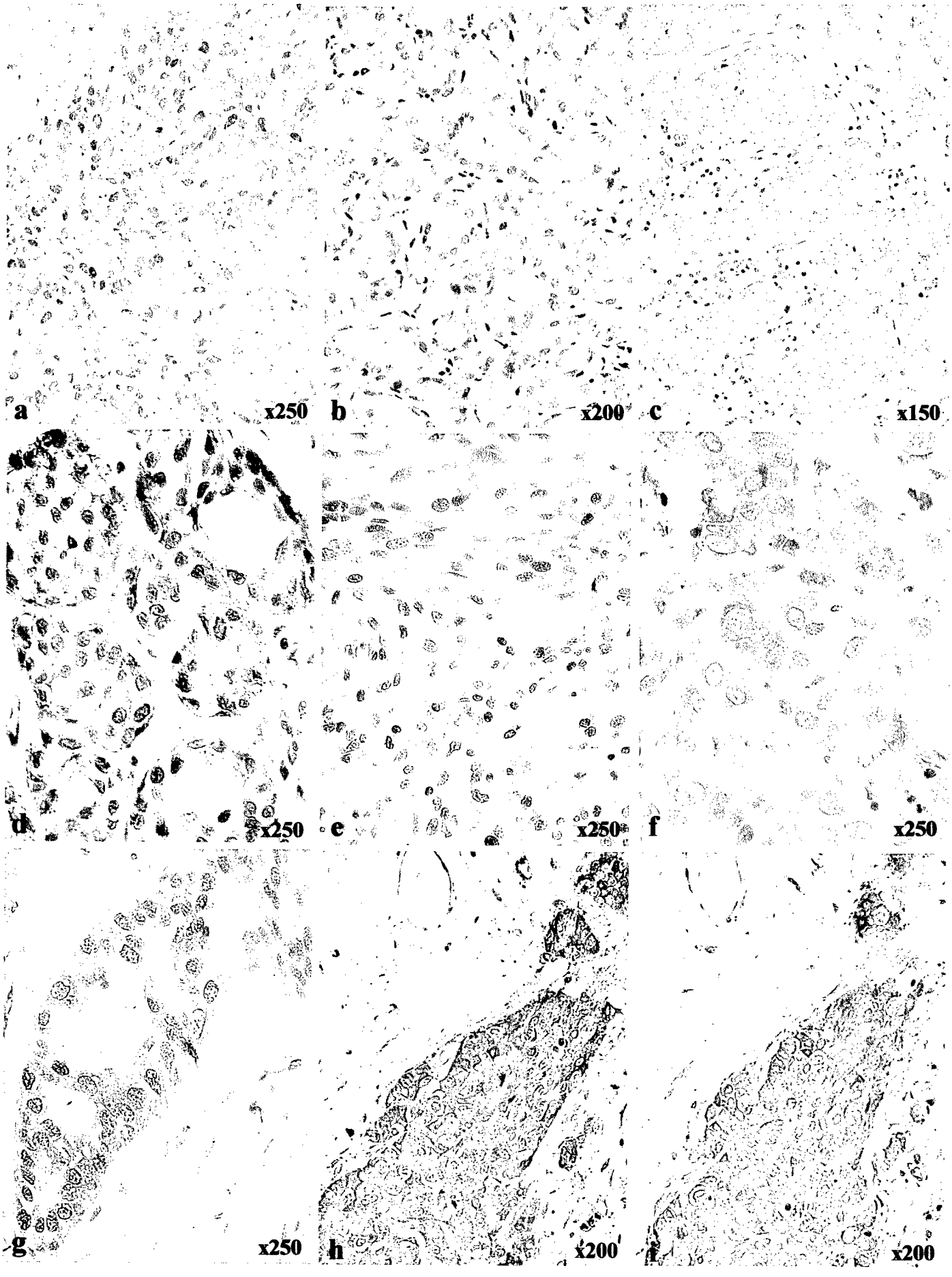

Figure 3

Appendix V

Topographic relationships among the expression of cyclins D1, E, and A2 and c-Myc in high-grade breast cancer

Dezhong Joshua Liao[1,2], Jan Blancato[3], and Robert B. Dickson[1,2,4]

[1]Lombardi Cancer Center, [2]Department of Oncology, [3]Institute for Molecular and Human Genetics, [4]Department of Cell Biology, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington, DC 20007

Running title: **Expression of cyclins in breast cancer**

Key words: **c-*myc*, cyclin D1, cyclin E, cyclin A2, breast cancer**

Correspondence:

Dr. Robert B. Dickson, Research Building, W417, 3970 Reservoir Road, NW, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007

Tel: (202) 687-3337

Fax: (202) 687-7505

Email: DicksonR@gunet.georgetown.edu

Abstract

c-Myc oncoproteins can initiate transformation of cultured cells and mammary carcinogenesis of transgenic mice. These functions may be exerted partly via regulation of expression of several putative target genes of c-Myc, including cyclins D1, E and A2. Our previous observation in the MMTV-c-*myc* transgenic mice showed that c-*myc*-initiated mammary tumors were initially positive for cyclin A2, but then underwent focal inactivation of expression of c-*myc* and cyclin A, coincident with expression of cyclins D1 and E. Conversely, in c-*myc*/*tgfα* bitransgenic mouse mammary tumors, all three cyclins and c-Myc are concomitantly expressed. Few studies have addressed the interrelationships among expression of c-Myc and these cyclins in human cancers, due to the limitation in the approaches of manipulating gene expression in a human tissue. In the present study, we examined the topographic relationships of expression of these three cyclin genes and c-Myc on serial sections. In the majority of tumor areas examined, cyclin D1 and c-Myc either were reciprocally expressed, or were localized in different subcullular compartments, i.e. the cytoplasm vs nucleus. Expression of cyclin E was associated with expression of cyclinD1, c-Myc, or both. Expression of cyclin A2 was very heterogeneous and did not seem to be associated particularly with c-Myc nor cyclins D1 and E. Nevertheless, cyclin A2 labeling indices were statistically associated with the Ki67 labeling indices in most, but not all, tumor areas, indicating that expression of cyclin A2, in general, reflected the proliferation of tumor cells. Invasive tumor cells that were disseminated in the stromal or fat tissue usually manifested a concomitant nuclear staining of c-Myc and cyclins D1 and E, but not cyclin A2. Taken together, these results suggest, indirectly, that c-Myc and cyclin D1 may be reciprocal in their expression or subcelluar localization, whereas expression of cyclin E may require either cyclin D1 or c-Myc. Concomitant expression

of c-Myc and cyclins D1 and E in the nucleus may reflect a more invasive potential than expression of each of these genes alone. Expression of cyclin A2 may reflect tumor cell proliferation in general, but it is not yet established as a surrogate of proliferation marker for breast cancer.

Introduction

Breast cancer and many other types of malignancy exhibit overexpression of several genes, including c-*myc* and cyclins D1, E and A2, which function to drive cell cycle progression, consistent with deregulated cellular proliferation [1-23]. However, while a correlation among expression of these cell cycle regulators and cancer aggressiveness is indeed observed in many studies, the opposite correlation has also been shown frequently in other clinical reports, even for the same type of cancer [24]. For instance, although overexpression of c-Myc has been shown in some studies to associate with a better patient survival or a better tumor differentiation for breast cancer [25,26] and for cancer of the testis, bile ducts and colon, other studies show the opposite [24]. Similarly, cyclin D1 overexpression in breast cancer is reported to be associated with a poorer prognosis in some studies [27,28] but with a better tumor differentiation and a positive expression of estrogen receptor (ER) α in other studies [3,29,30]; ERα positivity is generally considered to be an indication of a better therapeutic response and patient outcome. These paradoxical data indicate that the relationship between aberrant expression of these genes and the tumor aggressiveness may depend also on certain yet undefined tumor parameters.

C-*myc* is an immediate early growth response gene and is expressed during G0/G1 transit of the cell cycle upon exposure of cells to extracellular growth stimuli, such as estrogen in the cases of mammary epithelia and breast cancer cells [24,31,32]. Expression of cyclin D1 is induced during mid G1 phase, also upon exposure to extracelular growth stimuli; it may remain high during S phase [33]. Expression of cyclin E rises at late G1 and extends to early S phase [33]. Unlike c-Myc and cyclin D1, expression of cyclin E is usually triggered by intracellular signals [34]; induction of cyclin E by extracellular growth signal may thus have an indirect mechanism.

Cyclin A2 expression starts at G1/S transit and is maintained at high levels through S, G2 and M phases to facilitate DNA replication and mitosis [35-37].

Cyclins D1, E and A2 have all been suggested as c-Myc target genes [24,38-40]. Expression of cyclin D1 has been shown to be activated by c-Myc in cultured fibroblast cell lines [39]. Induction of cyclin D1 has also been observed in liver cancer from mice in which a c-*myc* transgene was targeted to the liver [41]. Conversely, however, other *in vitro* experiments have also shown that c-Myc suppresses expression of cyclin D1 [42-44]. In our prior studies of mammary tumors developing from the mice carry a c-*myc* transgene under the control of mouse mammary tumor virus (MMTV) long terminal repeat, we found that cyclin D1 expression was localized exclusively in the specific focal areas that had lost expression of c-Myc, but not in the areas showing high levels of c-Myc. This reciprocal expression of c-Myc and cyclin D1 may suggest, indirectly, that c-Myc may suppress cyclin D1 in mammary tumors, at least under a transgenic situation [45]. A third relationship between c-Myc and cyclin D1 has also been postulated in the literature, which considers that expression of cyclin D1 is not regulated by c-Myc but, instead, parallels with the expression of c-Myc; these two proteins cooperate to facilitate cell cycle progression and to inhibit apoptosis [33,46-49]. Currently, it is unknown which of these three types of relationship between c-Myc and cyclin D1 really occurs *in vivo* in general and in human breast cancer in particular.

Expression of cyclin E has also been shown to be induced by c-Myc, but the induction is considered, at least by some investigators, to be an indirect event [32,38]. Mice in which the cyclin D1 gene is replaced with the cyclin E gene by a knockin approach show a phenotype identical to the cyclin D1 wild type, strongly suggesting that cyclin E may be the ultimate target of cyclin D1 responsible for the downstream events of cyclin D1 [34]. Therefore, it remains possible that cyclin

D1 may function as an intermediator for the upregulation of cyclin E by c-Myc or other extracellular growth signals, since cyclin D1 is expressed earlier than cyclin E in the cell cycle and since it can be induced by c-Myc in certain situations. If cyclin E is a target of cyclin D1 rather than of c-Myc, then in a system where cyclin D1 is suppressed by c-Myc, expression of cyclin E should not be observed. This is exactly what we have observed in the mammary tumors developing from MMTV-c-*myc* transgenic mice. In this tumor type, expression of cyclin E is always co-localized with that of cyclin D1, i.e. only in the focal areas that have lost c-Myc expression, but not in the tumor areas where c-Myc levels are high.

Transcription of cyclin A2 has been shown to be activated directly by c-Myc [38,40]. Also, it has been shown that expression of cyclin A2 can be induced by the cyclin E-cdk2 complex [50]. Therefore, at least theoretically, expression of cyclin A2 could be induced by c-Myc via sequential mediations of cyclin D1 and cyclin E as well, since cyclin E expression rises later than cyclin D1 but earlier than cyclin A2 in the cell cycle. It is unknown which of these two activation pathways occurs *in vivo* in general and in human breast cancer in particular.

We hypothesize that the paradoxes in the relationship between overexpression of c-*myc* or cyclin D1 on one side and the tumor aggressiveness on the other may be in part related to the interrelationships among c-*myc* and its target cyclin genes [24], since they are expressed sequentially from G0/G1 transit through G1, S, G2 and M phases. Although there have been many reports on the expression of these genes in human cancers, few studies have been carried out to explore the interrationships among these genes in a human cancer tissue, due to the limitation in the approaches of manipulation of gene expression under an *in vivo* situation. Relevant data are obtained mainly from *in vitro* experiments with cultured cells that ectopically express these genes. We therefore studied the interrelationships among c-Myc and its putative

target cyclins in high-grade breast cancer biopsies with an immunohistochemical approach. High-grade tumors are usually very heterogeneous in histology and tumor biology, which provides us with a unique approach to explore, in a topographic manner within individual tumors, whether the interrelationships among expression of c-Myc and its target cyclins are related to the tumor morphology.

Materials and Methods

Materials. Fifty-two cases of formalin-fixed, paraffin-embedded tissue blocks of biopsies of untreated human breast cancer were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Cancer Center at Georgetown University Medical Center. The criteria for tumor selection were the following parameters: negative progesterone receptor status, positive lymph node involvement, and high tumor grade. The parameters were chosen from our prior meta analysis as indication of a high likelihood of c-*myc* amplification [51]; a study focused on the amplification, RNA expression, and protein levels of the c-*myc* gene with these specimens will be separately reported elsewhere. Serial sections (5 μm) of individual tissue blocks were prepared by the Histopathology Laboratory.

Immunohistochemistry. Immunohistochemical staining was performed using an avidin-biotin complex (ABC) method described previously [45,52]. One serial section of each specimen was deparaffinized and blocked with 3% peroxide. Antigens were retrieved by heating in a microwave oven in 50 mM citrate buffer, pH 6.0, after boiling for 8 minutes. After blocking with 6% normal goat serum, the section was incubated with a primary antibody for 2 hours, followed by 1 hour incubation with a second antibody conjugated with biotin (Vector Laboratories Inc., Burlingame, CA). The section was then incubated with peroxidase-conjugated avidin (Dako

Corporation, Carpinteria, CA) for 30 minutes, followed by color development with diaminobenzidine and peroxide. All procedures were carried out at room temperature. The data presented were generated by using monoclonal anti-c-Myc antibody (9E10) from Sigma Chemical Company, St. Louis, MO, rabbit Ki67 (A0047) from Dako Corporation, Carpinteria, CA, and rabbit polyclonal cyclin D1 antibody (C19), rabbit cyclin E antibody (C19), and rabbit cyclin A2 antibody (H432) from Santa Cruz Biotechnology Inc., Santa Cruz, CA. For verification of the staining specificity, serial sections from at least 5-10 cases were also stained by using the following antibodies: Ki67 (NCL-Ki67p) from Vector Laboratories Inc., Burlingame, CA, antibodies of cyclin A2 (06-138), cyclin D1 (06-137), and cyclin E (06-459) from Upstate Biotechonology Inc., Lake Placid, NY, as well as antibodies of cyclin A2 (C19), cyclin D1 (C20), cyclin E (M20), and c-Myc (C19) from Santa Cruz Biotechnology Inc., Santa Cruz, CA. These antibodies gave rise to the signals similar to the aforementioned antibodies used to generate the data, but with different staining intensities. Also to control the signal specificity, serial sections were made from five selected positive cases and were subjected to the same staining procedure, with a normal mouse IgG to replace the primary antibody. This control staining did not give rise to a signal. The cut-off for positive cases was arbitrarily set at 10% or more of tumor cells that showed a clear staining.

Quantification of Labeling Indices. For each tumor sample, four areas randomly selected on serial sections stained for cyclin A2 and Ki67 were counted for the labeling indices. Totally 200 areas were counted. A pair of the same area stained for cyclin A2 and Ki67 are shown in fig 2H and 2I as an example. The numbers of positive and negative cells on each pair of areas were quantified. The percentages of positive cells were calculated by dividing the number of positive

cells by the whole number of cells counted. The paired scores were used to plot the scattergram and

Statistical analyses. The paired scores of cyclin A2 and Ki67 were calculated for the coefficient *r*. A Wilcoxon rank sum test was used for analysis the differences of Ki67 and cyclin A2 scores among categories of the relationships among expression of c-Myc and cyclins D1 and E, because the labeling indices varied greatly. χ^2 test was used for the comparisons among the percentages of different patterns of the interrelationships among expression of c-Myc and cyclins D1 and E. A p value of 0.05 was also used as the cut off for the significance of all these analyses.

Results

Expression of cyclin D1 and its relationship to c-Myc

About 75% (39/52) of the cases studied showed positive for cyclin D1. However, in about half of the positive cases, either the number of positive cells were as few as about 10%, or the staining intensity was weak. The staining in tumor cells was more commonly localized to the cytoplasm (fig 1A) than to the nucleus or to both cytoplasm and nucleus (fig 1E). Some fibroblasts in the stroma were also positive, but their staining was only in the nucleus. Normal mammary glands found in some tumor specimens were negative for cyclin D1. Hyperplastic lesions of mammary epithelia adjacent to the tumor areas, such as the lesion shown in fig 2 for cyclin A2, were either negative or weakly positive in the cytoplasm.

Tumor cells positive for cyclin D1 showed great intratumoral heterogeneity in most cases. Usually, within a tumor individual, some patches of tumor cells were positive, while some other patches were negative. We randomly examined 200 areas of tumor cells (4 areas per tumor sample) on serial sections stained for c-Myc and cyclins D1 and E to quantify the frequencies of

different patterns of the relationships among expression of these three genes. Negative staining and cytoplasmic staining of cyclin D1 and c-Myc were grouped into the same category because cyclin D1 [48,53] and c-Myc (as a transcription factor) function inside the nucleus. Cyclin E staining was categorized to only positive and negative since cyclin E nuclear staining in tumor cells was always found to be associated with cytoplasmic staining, as observed previously in mouse mammary tumors [45] and hamster renal tumors [52]. The percentages of these areas showing various relationships among expression of these genes were listed in table 1.

With regard to the relationship between expression of cyclin D1 and c-Myc, four patterns of relationships were observed: 1) negative c-Myc with nuclear cyclin D1 (fig 1D vs 1E), 2) positive nuclear c-Myc with negative or weak cytoplamsic cyclin D1 (fig 1G vs 1H), 3) strong cytoplasmic cyclin D1 with nuclear c-Myc (1A vs 1B), and 4) nuclear c-Myc with both nuclear and cytoplasmic cyclin D1 (fig 1J and 1K). Totally 32.5% (category 4 plus 8 in table 1) of the tumor areas showing nuclear staining of cyclin D1 (fig 1E) were negative for c-Myc (fig 1D). On the other hand, 42% of the areas (category 1 plus 5 in table 1) exhibiting nuclear staining of c-Myc showed either negative staining, or various extents of cytoplasmic staining, of cyclin D1 (fig 1G vs 1H). Therefore, totally 74.5% of tumor areas examined showed a reciprocal nuclear expression between c-Myc and cyclin D1. Concomitant expression of c-Myc (fig 1J) and cyclin D1 (fig 1K), which was usually positive for cyclin E positive (fig 1L), was observed only in the tumor areas where invasive tumor cells were disseminated individually in the stroma or fat tissue. This pattern occupied only 14% (category 3 plus 7 in table 1) of the tumor areas examined, much lesser than the 74.5% of the areas that showed reciprocal expression ($p<0.05$).

Expression of cyclin E and its relationship to cyclin D1 and c-Myc

About 56% (29/52) of the cases were positive for cyclin E, but in many positive cases the staining intensity were weak to moderate. In most cases, the staining was localized to both cytoplasm and nucleus in the areas with different morphology (fig 1C, 1F, 1I, and 1L). Some fibroblasts also were positive for cyclin E, and the staining was only in the nucleus. Normal mammary glands found in some tumor specimens were negative for cyclin E. Hyperplastic lesions adjacent to tumor areas, such as the lesion shown in fig 2 for cyclin A2 staining, were either negative or weakly positive in the cytoplasm.

Great intratumoral heterogeneity of cyclin E positivity was also observed, similar to the staining for cyclin D1. In most cases, the patches of tumor cells positive for cyclin E were associated with a positive nuclear staining of c-Myc (category 1 in table 1), cyclin D1 (category 4 in table 1), or both (category 3 in table 1), but not all of the patches that were positive for cyclin D1 or c-Myc showed cyclin E staining. As calculated from table 1, of total 113 areas (sum of categories 1 to 4 in table 1) that were positive for cyclin E staining, about 39% (category 1 in table 1) were also positive for c-Myc in the nucleus but negative or only cytoplasmic positive for cyclin D1 (fig 1B, 1A and 1C), and about 34% (category 4 in table 1) were positive for cyclin D1 in the nucleus but negative for c-Myc in the nucleus (fig 1E, 1D, and 1F). Only about 8% of cyclin E positive patches were negative for both c-Myc and cyclin D1 (category 2 in table 1).

Interestingly, there was 20% of total 200 areas examined showed nuclear c-Myc but negative for cyclin E (category 5 in table 1), indicating that cyclin E might not necessarily be induced when c-Myc existed in the nucleus. One logic explanation was that cyclin E expression in the majority of these tumor patches might be dependent on cyclin D1, since these areas showed nuclear negative for cyclin D1 and since the tumor patches showing negative nuclear c-Myc and cyclins D1 and E was as low as 7% (category 6 in table 1).

Expression of cyclin A2 and its relationship to c-Myc, other cyclins, and Ki67

Tumor cells positive for cyclin A2 were observed in all of the cases studied. The staining usually was in the nucleus of tumor cells, but concomitant cytoplasmic staining was also discerned in some tumor cells (fig 2A and 2B). Cytoplasmic staining alone was only observed in the mitotic cells that had no nucleus, and in the tumor cells that invaded into the stroma or fat tissue (fig 2C and 2D). Fibroblasts positive for cyclin A2 could be discerned only occasionally. Normal mammary glands seen in some tumor specimens (fig E) and hyperplastic lesions adjacent to the tumor areas (fig 2F) hardly exhibited positive cells.

In the tumors with squamous differentiation, cyclin A2 positive cells were confined to the basal layer (fig 2G), which couples cyclin A2 expression with the proliferating potential. Since mitosis is the only reliable marker for the proliferating cells, we carefully identified 100 mitotic cells under microscope and found that 97 of them were positive for cyclin A2. Since Ki67 was the most commonly used marker for proliferating cells in pathologic diagnosis, labeling indices of Ki67 and cyclin A2 were counted on paired-areas of tumor cells on serial sections; a representative pair of these tumor areas were shown in fig 2G and 2H. The labeling indices of cyclin A2 and Ki67 were positively correlated (fig 3), with an average labeling index of 14.6± 10.6% for cyclin A2 and 14.8±10.3 for Ki67. However, as shown in fig 3, some patches of tumor cells had much higher scores of Ki67 than cyclin A2 scores, while other patches showed the opposite. Hyperplastic lesions usually showed more Ki67 positive cells than cyclin A2 as well.

The number of cells positive for cyclin A2 showed the greatest intratumoral heterogeneity, compared to the staining for c-Myc, Ki67, and cyclins D1 and E. As exemplified in Fig 2A and 2B, the labeling index could be as high as about 35% in one area (fig 2A) but be lower than 2%

in another area of the same tumor (Fig 2B). Heterogeneity in the density of positive cells was even greater among different tumors, even when the tumors with similar histology were compared. The density of labeled cells did not seem to be related to any specific tumor morphology. However, the areas that showed a greater invasive potential, such as those shown in fig 2C and 2D, usually had a lower score of nuclear staining (category 3 in table 1), although most tumor cells in these areas usually manifested cytoplasmic staining.

Staining of cyclin A2 did not exhibit any association with the expression of c-Myc, cyclin D1, nor cyclin E (table 1), due largely to the great heterogeneity of the labeling indices. Cyclin A2 positive cells could be observed in areas that were either positive or negative for these proteins (table 1). For instance, the same patch of tumor cells shown in fig 1G, 1H and 1I, as well as the patch shown in fig 1J, 1K and 1L, contained very few cells with cyclin A2 nuclear staining (not shown), whereas the patch showing in fig 1A, 1B and IC, as well as the patch showing in fig 1D, 1E and IF contained relatively more cyclin A2 positive cells in the nucleus (not shown). However, in the areas where invasive tumor cells showed concomitant expression of c-Myc and cyclins D1 and E, the cyclin A2 index, scored by only nuclear staining, was significantly lower than that of Ki67 (category 3 in table 1).

Discussion

Several studies have shown that the expression of cyclin D1 in breast cancer is associated with the ERα positivity and better-differentiated tumors [1,3], whereas other studies show the opposite correlation [27,28], no correlation [10], or correlation only with node-positivity [11]. On the other hand, expression of cyclin E has been reported in several studies to associate with ER negativity, greater tumor aggressiveness, and poorer prognosis [7,8,15,21]. By suing a western blot

approach, Nielsen *et al* further show that expression of cyclins D1 and E is reciprocal in the same breast cancer biopsies [54]. The present study shows that cyclin D1 expression can be detected in the majority of high-grade breast biopsies, but in most cases the staining intensity is weak and the number of positive tumor cells is relatively low. It is possible that low-grade tumors with ER positivity may show a stronger staining intensity and a higher percentage of positive cells. In addition, our samples show a high percentage of cyclin E positivity, which is in general consistent with the results of Nielsen *et al* [19]. The main difference is that by comparisons of the same patches of tumors on serial sections stained for these two cyclins, we observed both concomitant and reciprocal expression of cyclin D1 and cyclin E.

By comparison of the same patches of tumor cells on serial sections, we found four patterns of relationship between c-Myc and cyclin D1. As exemplified in figure 1D vs 1E and 1G vs 1H, expression of c-Myc and cyclin D1 in the majority of tumor patches examined is reciprocal, similar to what was observed in the mammary tumors from c-*myc* transgenic mouse [45]. These findings may indirectly support the notion that c-Myc may suppress expression of the cyclin D1. However, it is also observed that cyclin D1 and c-Myc are co-localized to the same patches of tumor cells, as seen in figure 1A vs 1B, indicating that the expression of cyclin D1 may not be suppressed by c-Myc under certain situations. Nevertheless, their different subcellular localizations, i.e. nucleus vs cytoplasm, suggest a functional reciprocity between these two proteins, since cyclin D1 needs to be transported to the nucleus in order to be functional [48,53], whereas its phosphorylation at the cytoplasm facilitates its degradation [55,56]. Possibly, this is also one of the reasons for the paradoxical relationships between cyclin D1 expression and clinicopathological parameters in the literature, since most studies do not deal with cytoplasmic and nuclear cyclin D1 separately.

Another pattern of the relationship between c-Myc and cyclin D1 is their concomitant existence in the nucleus, usually together with cyclin E, as shown in figure 1J, 1K and 1L, which occpies 11% of the tumor areas examined. We have observed the similar pattern in the mammary tumors from mice carrying both *tgfα* and c-*myc* transgenes [45]. In these double transgenic tumors, cyclin D1 is induced, most likely by TGFα, and concomitant with the expression of c-Myc. Cyclin E is also expressed and co-localized with cyclin D1 in the double transgenic tumor. This concomitant expression of c-Myc and G1 cyclins is coupled with more proliferation but less programmed cell death and may account for the earlier development of the tumor, compared to the c-*myc* transgenic mammary tumor [24,45]. Human breast tumor cells, especially those that have invaded to, and are sparsely disseminated in, stromal or fat tissue, may be under stronger influences of stromal-tumor interactions and humeral factors, such as many growth/survival-promoting growth factors and estrogens that have a role similar to TGFα. These special situations may be the reasons for the concomitant localization of c-Myc and G1 cyclins in the nucleus of invasive tumor cells, and confer these tumor cells more aggressiveness.

Both c-Myc and cyclin D1 are G1 progression-driving factors delivering extracellular growth signals to the cell. A common downstream event of these two proteins to drive G1 progression may be the recruitment of cyclin E [57]. In the present study, nuclear localization of cyclin D1 is found to be associated with nuclear staining of cyclin E in about 34% of the tumor areas that were positive for cyclin E but negative for c-Myc staining. On the other hand, 20% of the total tumor areas examined that showed nuclear c-Myc but negative or only cytoplasmic positive for cyclin D1 are still negative for cyclin E. These results are in line with the concept that cyclin E expression may require functional cyclin D1 in many human breast cancer biopsies. However, in about 39% of tumor cells that were positive for cyclin E where c-Myc is expressed

while cyclin D1 is either negative (fig 1G vs 1H) or localized to the cytoplasm (fig 1A vs 1B), expression of cyclin E can still be detected (fig 1C and 1L). These latter results indicate a possibility that under certain yet undefined situation, cyclin E may still be induced by c-Myc, which is different from what we observed in the c-*myc* transgenic mammary tumors [45]. *In vitro* experiments have shown that expression of cyclin E without activation of cyclin D1 is likely to bypass the cyclin D/cdk4/p16/pRB feedback loop to drive the tumor progression [12].

Few references have described the immunohistochemical properties of cyclin A2 in human breast cancer, although several studies involve immunohistochemistry of cyclin A2 [4,9]. The positive correlation between cyclin A2 scores and Ki67 scores observed in the present study, suggest that cyclin A2 expression may reflect cell proliferation, a conclusion also reached by studies of other types of cancer [58-61].

However, several findings in the present study raise a question that is not answered by this nor other reported studies as to whether cyclinA2 expression drives the proliferation characteristics of certain tumor types or is merely a reflection of proliferation. First, similar to what was reported for head and neck cancer [62], we observe that cyclin A2 scores are much higher than Ki67 scores in several patches of tumor cells, as shown in figure 3. It is likely that in these patches of tumor cells, cyclin A2 is overexpressed, and that the overexpression is disassociated from proliferation. Second, conversely, figure 3 also shows that the cyclin A2 scores are much lower than the Ki67 scores in several other tumor patches, implying that the proliferating potential may be disassociated from the expression of cyclin A2. This property seems to appear in the hyperplastic lesions as well, where cyclin A2 positive cells are hardly discerned (fig 2F), although hyperplastic lesions do proliferate by the definition and by the observation of Ki67 positive cells. Third, in some invasive areas as shown in fig 2C and 2D, cyclin A2 is actually

expressed but is confined in the cytoplasm. These invasive tumor cells are likely to be at G1 phase, since they show also expression of c-Myc and cyclins D1 and E and since cyclin A2 enters the nucleus at the beginning of S phase [36,37]. It is possible that the cells with strong invasive potential may be confined at G1 phase and may have less proliferating potential. Thus, the invasive potential may be disassociated with the proliferating potential, at least under certain circumstances. Nevertheless, these several observations lead to a consideration that, unlike Ki67, expression and function of cyclin A2 may be aberrantly regulated in certain breast cancer cells and may reflect certain yet undefined tumor characteristics other than proliferation.

In summary, c-Myc vs cyclin D1, and cyclin D1 vs cyclin E, may have a reciprocal relationship in their expression and/or functions in some patches of tumor cells but a concomitant relationship in other patches. The invasive tumor cells that were disseminated sparsely in the stromal or fat tissue usually showed concomitant nuclear localization of c-Myc and cyclins D1 and E, but not cyclin A2. Cyclin A2 labeling scores were statistically correlated with the Ki67 scores, and thus reflect in general proliferation of tumor cells. However, to consider cyclin A2 as a surrogate of proliferation marker for breast cancer will require more study, since it remains possible that expression and functions of cyclin A2 may be altered in certain tumor cells and such alterations may reflect certain tumor characteristics other than proliferation.

Acknowledgements

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Fig legends:

Fig 1: Immunohistochemical staining for c-Myc and cyclins D1 and E. An area of invasive tumor cells showed strong cytoplasmic staining of cyclin D1 (A), whereas many tumor cells at the same area showed nuclear staining of c-Myc (B) and both nuclear and cytoplasmic staining for cyclin E (C). In another patch of tumor cells, negative staining of c-Myc (D) is associated with strong nuclear staining of cyclin D1 (E), and both cytoplasmic and nuclear staining of cyclin E (F). However, when many tumor cells showed nuclear staining of c-Myc (G), they manifested only weak cytoplasmic or perinuclear staining of cyclin D1 (H) and strong staining of cyclin E in either cytoplasm or both cytoplasm and nucleus (I). In the area where invasive tumor cells were disseminated sparsely in the fat tissue, concomitant expression of c-Myc (J), cyclin D1 (K) and cyclin E (L) in the nucleus was discerned.

Fig 2: Immunohistochemical staining of cyclin A2 and Ki67. The staining was localized to the nucleus in most tumor cells (A and B). The number of positive cells varied greatly, with a very high density of positive cells in an area (A) but a much lower density in another area of the same tumor (B). In two different tumors (C and D), tumor cells invading to the stroma showed mainly cytoplasmic staining of cyclin A2 with few cells showing nuclear staining. Normal mammary glands (E) found in a tumor specimen were negative for cyclin A2, whereas a hyperplastic lesion (F) adjacent to the tumor areas (up-right corner) showed only one positive cell stained in the nucleus (arrow). Positive staining in a tumor tissue with squamous differentiation (G) was localized to the nucleus of the cells in the basal layer. An area of cyclin A2 staining (H) and its

corresponding area of Ki67 staining (I) on a serial section showed the relationship between expression of cyclin A2 and Ki67.

Fig 3. Scattergram showing correlation between cyclin A2 and Ki67 staining in paired areas on serial sections. Note that although the correlation is statistically significant ($p<0.05$), some areas of tumor cells show much higher sores of cyclin A2 than Ki67 scores, and some other areas show the opposite.

Table 1. Percentages of tumor areas showing different staining patterns, Ki67 scores, and cyclin A2 scores.

Cat.	Staining[a]	No of Areas	(%)[b]	Ki67(%)[c]	A2[d]
1	M-N, D-C/-, E+	44	22.0	16.3±9.3	16.5±9.8
2	M-C/-, D-C/-, E+	9	4.5	15.2±10.3	14.9±9.9
3	M-N, D-N, E+	22	11.0	9.3±6.8[e]	5.9±4.3[f]
4	M-C/-, D-N, E+	38	19.0	15.7±9.5	15.9±10.7
5	M-N, D-C/-, E-	40	20.0	14.4±8.8	12.1±7.4
6	M-C/-, D-C/-, E-	14	7.0	9.7±6.7	7.8±5.6
7	M-N, D-N, E-	6	3.0	11.6±7.9	10.3±6.8
8	M-C/-, D-N, E-	27	13.5	12.0±7.4	10.8±8.2

Note:

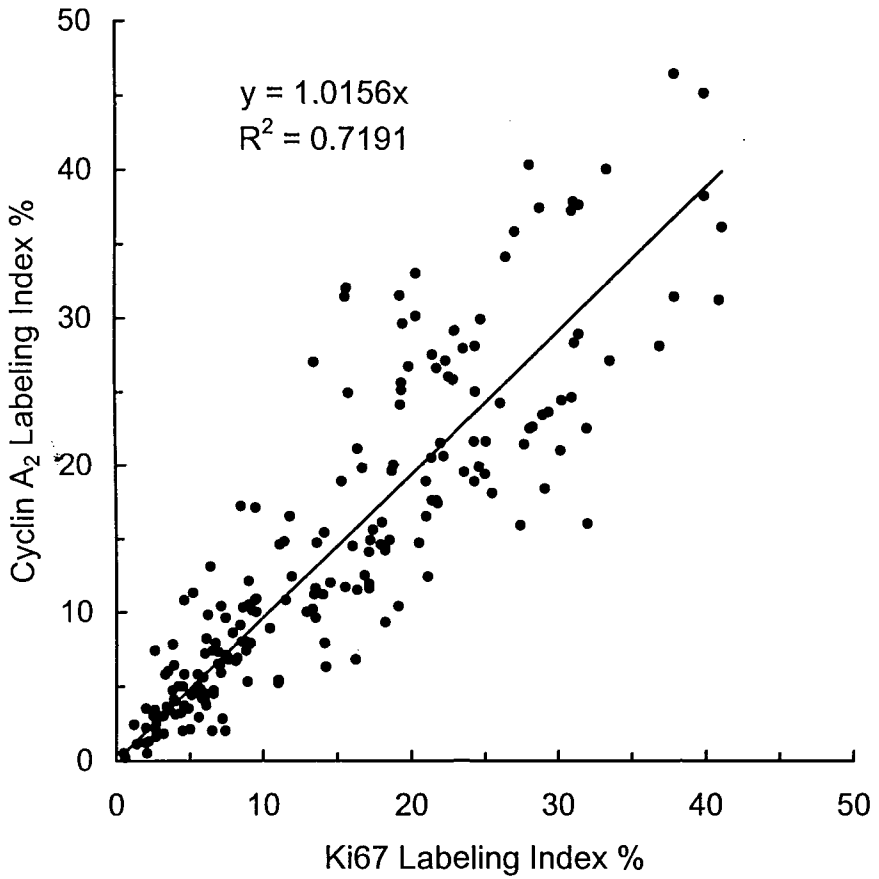
a: Staining patterns in categories 1 to 8 are: 1. c-Myc nuclear positive, cyclin D1 negative or positive only in the cytoplasm, and cyclin E positive; 2. c-Myc negative or positive only in the cytoplasm, cyclin D1 negative or positive only in the cytoplasm, and cyclin E positive; 3. c-Myc, cyclin D1 and cyclin E all positive in the nucleus; 4. c-Myc negative or positive only in the cytoplasm, cyclin D1 negative or positive only in the cytoplasm, and cyclin E positive; 5. C-Myc positive in the nucleus, cyclin D1 negative or positive only in the cytoplasm, and cyclin E negative; 6. C-Myc negative or positive only in the cytoplasm, cyclin D1 negative or positive only in the cytoplasm, and cyclin E negative; 7. C-Myc positive in the nucleus, cyclin D1 positive in the nucleus, and cyclin E negative; 8. C-Myc negative or positive only in the cytoplasm, cyclin D1 positive in the nucleus, and cyclin E negative.

b: The percentages (mean±S.D.) were calculated from totally 200 patches of tumor cells on serial sections stained for c-Myc, cyclin D1 and cyclin E.

c: The percentages (mean±S.D.) were calculated from the tumor areas within the categories.

e: Significantly higher than the cyclin A2 index in the same category ($p<0.05$).

f: Significantly lower than the percentages of categories 1, 2, and 4 ($p<0.05$% without corrections for the multiple comparisons).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Ki67 Labeling Index %
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20
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40
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Fig. 3

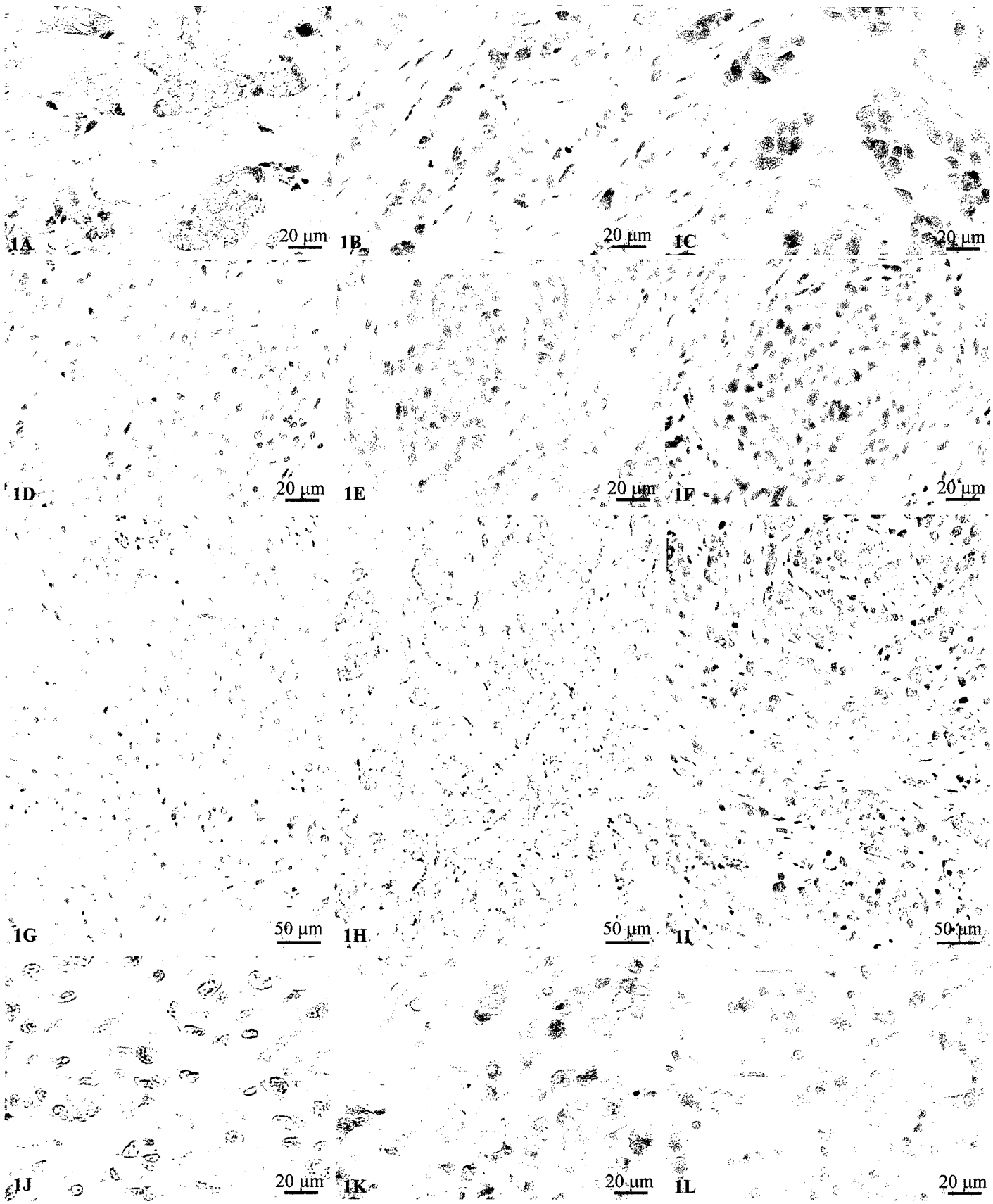
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Figure 1

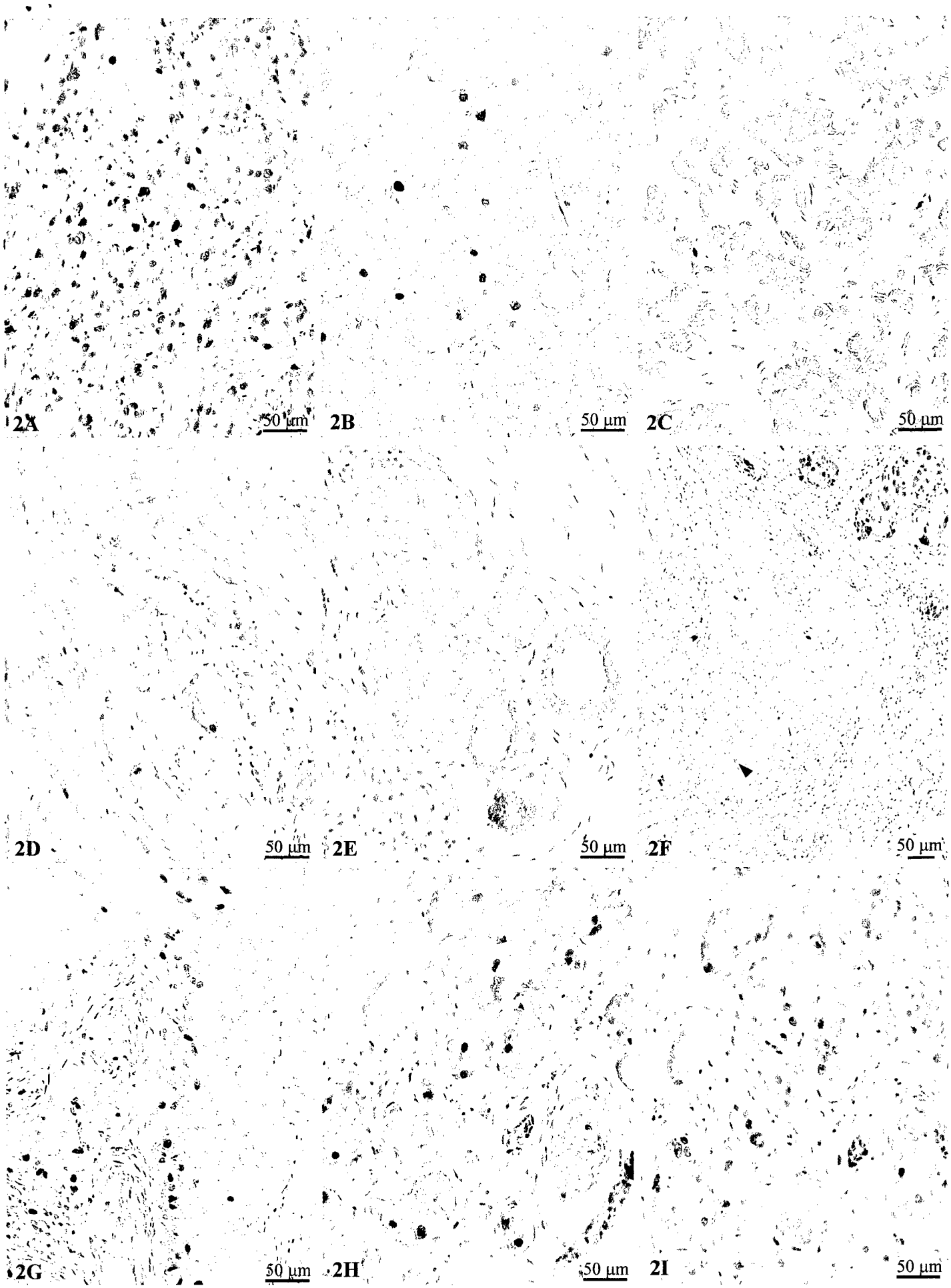
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Figure 2

Appendix VI

In press:
Proliferation of Normal and Malignant Mammary Epithelial Cells ---A special issue of
Journal of Steroid Biochemistry $ Molecular Biology

Guest-edited by **Robert B. Dickson** and **Dezhong Joshua Liao**

Roles of Androgens in the Development, Growth, and Carcinogenesis of the Mammary gland

Dezhong J. Liao and Robert B. Dickson
Oncology Department and Lombardi Cancer Center
Georgetown University Medical Center
Research Building, W416
3970 Reservoir Road, NW
Washington, DC 20007
Tel: (202)687-2277
Fax: (202)687-7505
Email: liaod@gunet.georgetown.edu

Abstract

Androgens influence the development and growth of the mammary gland in women. Treatment of animals and cultured cells with androgens has either inhibitory or stimulatory effects on the proliferation of mammary epithelia and cancer cells; the mechanisms for these dual functions are still not very clear and are discussed in this review. Epidemiological data suggest that, similar to increased estrogens, elevated androgens in serum may be associated with the development of breast cancer. Experiments in rodents have also shown that simultaneous treatment of androgen and estrogen synergizes for mammary gland carcinogenesis. Similar synergistic effects of both hormones have been observed for carcinogenesis of the uterine myometrium of female animals and for carcinogenesis of the prostate and deferens of males. There are also clinical and experimental indications for a possible association of elevated levels of both androgens and estrogens with the development of ovarian and endometrial cancers. A hypothesis is thus proposed that concomitant elevation in both androgens and estrogens may confer a greater risk for tumorigenesis of the mammary gland, and probably other female reproductive tissues than an elevation of each hormone alone.

Introduction

Similar to estrogens, androgens influence the functions of many organs in women, such as the hypothalamus-pituitary-ovary axis, mammary gland, uterus, bone, cardiovascular system, etc [1;2]. Data accumulated, especially during the recent years, have increased the attention to the adverse impact of subnormal levels of androgens on women's health. This raised attention has in turn led to an increase in the use of androgens to correct various clinical symptoms caused by androgen deficiency [1-3]. On the other hand, many lines of evidence have also shown that supernormal levels of androgens may also have adverse effects on the female reproductive system, including abnormal growth and possibly tumorigenecity [4;5]. This latter aspect has commanded much less wariness in the past and should be further studied, particularly because of the increasing use of androgens for various therapeutic purposes in women. This review summarizes mainly the experimental and clinical data on the effects of elevated androgens on the mammary gland, although data on the ovary and uterus are also discussed.

Uses of androgens in women

In clinics, many women, both before and after menopause, may have symptoms of androgen deficiency [1;2;6], including unexplained fatigue, lack of well-being, and diminished libido, although these syndromes are not specific for androgen deficiency. Androgens are often prescribed to correct the deficiency [2]. Androgens are also included in certain regimens of hormone replacement therapy for some postmenopausal women to improve their sexuality [7-11]. The additional androgen in hormone replacement therapy is even more important for the women who have received bilateral oophorectomy for any therapeutic reasons, because ovaries provide approximately half of the circulating testosterone (T) in premenopausal women [12;13]. Bone loss in both premenopausal and postmenopausal women has been reported to be associated with lower levels of total and free T [14]. In female monkeys and rats, T treatment results in increases in intrinsic bone strength and resistance to mechanical stress, as well as increases in bone mineral density, bone torsional rigidity and bending stiffness [15;16]. Treatment with androgen plus estrogen has been reported to have a better effect than estrogen alone to prevent bone loss in women and in female monkeys and rats [13;15-17].

Androgens alone may promote atherosclerosis, which was once a concern for using androgens for cardiovascular aspects in women. However, recent findings indicate that combined androgens and estrogens have the opposite effect in the arterial wall, as androgens help maintain

vasodilation [2;18;19]. These new findings support the use of combined estrogen and androgen in postmenopausal women from the cardiovascular consideration [19]. Moreover, women are much more predisposed than men to the development of autoimmune diseases, indicating a prophylactic role of androgens for these conditions [20;21]. Laboratory experiments reveal that androgens suppress both cell-mediated and humeral immune responses [2;20;21]. Treatment of postmenopausal women with T or dehydroepiandrosterone (DHEA) has been reported to improve the symptoms of rheumatoid arthritis[22].

Other potential indications for therapeutic uses of androgens in women include postmenopausal loss of muscle mass, management of wasting in HIV infection, and premenstrual syndrome [1;2;13]. Probably the more problematic use of androgens in women is for female-to-male transsexuals, because the doses of androgens prescribed for this purpose are usually higher and the treatment periods are much longer, compared to other therapeutic uses mentioned above. In addition, androgen abuse also occurs occasionally among some female athletes; these supernormal levels of androgens may cause adverse effects as well [23].

Role of androgens in the development and growth of the mammary gland in rodents

All newborn rats and mice have female-type of mammary glands, regardless of their genetic sex [24-27]. Male mice and rats lack nipples and their mammary glands are not connected to the skin [24-28]. Gonadectomy at an early age [25;26;29] or treatment with an androgen inhibitor during pregnancy [30] prevents the destruction of the primary duct in the fetal male mouse, leaving the primary duct and the glands connected to the skin in the adults. Conversely, female offspring of rats receiving T during pregnancy show abnormal development of nipples [31;32]. Androgens administered during early pregnancy of rats and mice prevent the formation of the mammary anlagen in the fetus, while injection of androgens into rats and mice during late pregnancy masculinizes the rudiments of mammary glands in the female offspring, with postnatal hypertrophy seen in some glands [33;34]. These prenatal masculinizing effects can be reversed by injection of the mother with antiandrogens [34;35]. When explanted *in vitro* and protected from androgenic influences, the mammary gland rudiments isolated from a 13-day-old male rat develop into a female-type gland [36]. Conversely, when female rudiments are cultured either together with testicular explants or in the presence of T, they become the male type [25;33]. All these data demonstrate that the environment of sex hormones during pregnancy and neonatal life determines the pattern of the mammary gland in adult life, regardless of the genetic sex. The influence of T on the mammary rudiment is more pronounced during the earlier fetal stage and involves not only the mammary epithelium but also stromal-epithelial interactions [37-41]. After 15 days following conception, the rudiment becomes less sensitive [36;42], but androgens can still inhibit the growth and the development of the mammary gland in later life [29;43]. Short-term, low doses of several forms of androgens have been shown to inhibit the estrogen-induced proliferation of the mammary epithelial cells in female rats, mice and monkeys during adulthood [44-46]. Similar inhibitory effects are also observed in cell or tissue culture [47;48].

In conflict with the above-described inhibitory effects of androgens, many references since 1936 have also shown that administration of androgens, usually at moderate to high doses, stimulates lobule-alveolar development and milk secretion in the mammary glands of female rats with an intact pituitary gland [49-54]. In male rats, this effect is much less pronounced; male rats receiving testosterone propionate (TP) develop mainly apocrine metaplasia, with the appearance of a large number of apoptotic cells [55]. However, large doses of TP given for long periods induce cystic formations of the mammary epithelia in the rat, and males seem to be more susceptible than females to this effect [49;51]. In contrast, female rats are more susceptible than males to the cyst induction by estrogens [49]. The growth stimulation can also be observed in

cultured cells from normal, atypically hyperplastic, and malignant mammary epithelia [56;57]. These effects of androgens both *in vivo* and *in vitro* may not be exerted via enzymatic conversion of androgens to estrogens, since an estrogen receptor antagonist cannot block the lobular-alveolar induction by T, DHT (dehydrotestosterone), or DHEA, and since DHT is a non-aromatizable androgen [52;56]. In addition, androgens have also been shown to inhibit differentiation of mammary glands *in vitro* [52;58], which may not be surprising, as growth stimulation is usually coupled by differentiation inhibition.

In hypophysectomized female rats, T induces a thickening and dilation of the mammary duct systems by proliferation and hypertrophy of the ductal epithelium, without lobule-alveolar formation [49]. Addition of growth hormone restores the induction of lobule-alveolar development by T in hypophysectomized rats, indicating that growth hormone is the major pituitary hormone required for facilitating the effects of androgen on lobular-alveolar induction [49].

Role of androgens during adulthood on mammary tumor development and growth

Mammary carcinogens such as 7,12-dimethylbenz(a)anthracene (DMBA), 3-methylcholanthrene (MCA) and methylnitrosourea (MNU) induce mammary tumors at an incidence of virtually 100% in female rats and mice but at virtually 0% in males, if they were given at a single dose [59]. Dao *et al* showed that in the rat, the incidences of DMBA-induced mammary carcinomas in non-castrated males, castrated males, non-castrated males receiving an ovarian graft, and castrated males receiving an ovarian graft were 0%, 14%, 16%, and 66%, respectively [60]. These data, together with many others, indicate that endogenous androgens may be prophylactic against DMBA-induced mammary carcinogenesis, while ovarian hormones are promotional [59-61]. On the other hand, Yoshida *et al* showed that multiple, biweekly intragastric intubations of DMBA induced mammary carcinomas in 100% of male and 84% of female rats that were gonadectomized at 27 days of age [62]. These results suggest a likely possibility that the cancer prophylaxis by androgens may be overridden by multiple doses of DMBA. Administration of DHEA to ovariectomized rats inhibits the growth of tumor xenografts formed by the human breast cancer cell line ZR-75-1 [63]. Various forms of androgens given to adult female rats inhibit mammary tumor induction by DMBA, MNU, or several types of estrogens [53;54;59;61;64-68]. Several forms of androgens given to adult female rats bearing palpable mammary tumors induced by chemical carcinogens cause regression of the tumors, although the regression may be incomplete in some cases [54;59;68-71]. Administration of T increases the latency of mammary tumor induction by estrogen when given before the tumor formation [72]. T also causes tumor regression when given to the rats bearing estrogen-induced mammary tumors, but the regression is only temporary, and the tumors will later become refractory to the androgen, if the animals continue receiving estrogen [72;73].

It has been shown in several experiments that while most chemical- or estrogen-induced mammary tumors respond to androgen administration with regression, there are always a few tumors showing a stimulatory response [59;73;74]. Notably, several studies have shown that administration of different forms of androgens at moderate or high doses to rats bearing chemical-induced mammary tumors enhances the tumor growth [59;75-77]. In addition, androgen treatment plus ovariectomy is less inhibitory than ovariectomy alone for the growth of DMBA-induced mammary carcinomas, indicating that androgens may counteract the inhibitory effect of ovariectomy [78]. Boccuzzi *et al* also show that DHEA stimulates the growth of the DMBA-induced mammary tumors in ovariectomized rats, although in their experiments DHEA inhibits the tumor growth in ovarian-intact rats [79]. The mechanisms for these stimulatory effects of androgens still remain unknown, although the phenomenon has been discerned for a few

decades. The conjecture that conversion of androgens to estrogens accounts for the stimulatory response is certainly possible, but this may not be the dominant event, as it does not explain why in many other experiments androgens are inhibitory rather than stimulatory, as described above paragraph. Clinically, treatment of breast cancer patents with androgens has occasionally been observed to exacerbate the tumor as well [74;80].

Role of prenatal and neonatal androgens in mammary gland carcinogenesis during adulthood of rodents

Both rats and mice of either sex exposed to sex steroids in utero or the neonatal period exhibit permanent functional alterations in the endocrine and reproductive systems [81-86]. These animals have also been shown to develop dysplasia and neoplasia in vaginal and endometrial epithelia, as well as in mammary glands in certain strains of mice [83;86-89]. Female rats neonatally exposed to a single dose of TP show a significantly lower mitotic rate of the mammary epithelia during adulthood [90]. However, treatment of these neonatally androgenized female rats with DMBA at day 52 of age induces a higher mitotic rate of the mammary epithelia before the tumor formation, compared to the non-androgenized counterparts [90].

Male and female mice neonatally exposed to either E2 or T exhibit increased incidences of mammary dysplastic lesions and carcinomas following their infection with mouse mammary tumor virus (MMTV) or their exposure to DMBA during adulthood; neonatal androgenization is more potent than neonatal estrogenization for this effect [86;91]. However, the influence of neonatal androgenization in DMBA-induced mammary carcinogenesis may be different in the rat. Some reports show that neonatal androgenization of female rats actually suppresses the induction of mammary adenocarcinomas by DMBA, while it induces mammary dysplasia, a lesion that is borderline between benign and malignant [92-97]. While some other studies in the rat confirm the increased induction of dysplastic lesions or adenofibromas (a benign form of tumor), they do not show a pronounced change in the incidence of the malignant tumors [86;90]. The reason for this species difference still remains unknown.

Verhoenven et al performed gonadectomy to neonatal male and female rats and immediately treated some of the animals with a single dose of T or E2 [98]. These animals did not show significant change in the incidence of DMBA-induced mammary carcinomas, compared to the gonadectomized rats without treatment of T or E2. Thus, it seems that the effect of neonatal androgenization or estrogenization on the induction of mammary carcinomas by DMBA may require intact gonads during neonatal life [94;99;100]. Christakos *et al* showed that DMBA given at day 55 of age failed to induce mammary tumors in neonatally androgenized and ovariectomized rats, but induced the tumors in neonatally androgenized, ovary-intact rats with a longer latent period compared to non-androgenized, ovarian-intact controls, again showing an inhibitory role of neonatal androgenization and a dependence on the ovary. Yoshida *et al* confirmed these findings of Christakos *et al* and further showed that injection of progesterone restored tumor induction in ovariectomized rats [99], thus pointing to progesterone as the hormone responsible for the ovarian dependence.

Yoshida *et al* [101] treated neonatally androgenized female rats with a single dose of DMBA on day 50 of age, and performed ovariectomy to one half of the rats 28 days after dosing, with or without simultaneous administration of E2 or progesterone. While about 90% of the androgenized, ovarian-intact rats develop mammary dysplasia (mastopathia cystica), only 4% of androgenized, ovariectomized rats develop the dysplasia. Dysplasia developed at 96% incidence in the rats receiving ovariectomy plus E2, in strong contrast to its 0% incidence in the rats receiving ovariectomy plus progesterone. These data suggest that in neonatally androgenized female rats, development of dysplasia is dependent on estrogen, while progesterone may be

either ineffective or inhibitory, in conflict with the progesterone-dependence of the effects of neonatal androgenization on malignant carcinoma induction by DMBA. The reason for this conflict is currently unknown. Since various progestins are known to have variable degrees of androgenic effects [76;102-105], it cannot be excluded that the diverse effects of progesterone reflect the possibility that normal, benign, and malignant mammary tissues may have differential responses to the progestational and androgenic effects of progesterone.

Although both neonatal estrogenization and androgenization depress ovarian functions, only neonatal androgenization has been shown to induce lactational alterations in female rats, suggesting the involvement of prolactin [49-51]. Indeed, increased levels of circulating prolactin have been reported in neonatally androgenized female mice and rats [94;106]. Prolactin is considered to be responsible for the ovarian-dependence of the effects of neonatal androgenization on DMBA-induced mammary carcinogenesis as well [94;106], since secretion of prolactin by the pituitary is controlled by the ovary. This consideration gains support from the fact that prolactin exerts a considerable influence on chemically induced mammary carcinogenesis [107-109]. Moreover, several *in vitro* studies also show that androgens induce expression of the prolactin receptor [110] as well as stimulate expression and secretion of prolactin in uterine stromal cells [111]. Thus, the lactational alterations in neonatally androgenized female rats may be due to increased function of prolactin-prolactin receptor signaling.

Indications for an association of androgens with breast cancer in women

Serum T concentration is ten times higher than that of E2 in women [112;113]. Both normal and cancerous breast tissues contain and produce several forms of androgens as well [114-123]. Androgen receptor (AR) is expressed in normal mammary epithelial and stromal cells [124;125]. Many pathologic studies have also demonstrated that over 70% of human breast cancer biopsies from untreated women are positive for AR; the percentages are usually higher than, or equal high to, the percentages of ER and PR positivity [126-135]. These facts provide the basis for a direct AR-mediated action of androgens in the normal and malignant breast tissues. On the other hand, several key enzymes responsible for metabolic conversions of various forms of androgens to estrogens have also been detected at significant amounts in the tissues of normal breast and breast cancer [122;136-141], which provides the basis for the local biotransformation of androgens to estrogens, resulting in estrogen excess [4;119;140;142-147]. Currently it still unclear whether the direct, androgenic function, or the indirect, estrogenic function is the major mechanism used by androgens to influence the growth of the mammary gland and mammary carcinoma.

Many references have documented higher T levels in urine and blood of pre- and post-menopausal breast cancer patients, with or without a concurrent increase in circulating levels of estrogens, compared to the normal women at the same ages [135;141;148-169]. However, some other studies show that the increased T occurs only in postmenopausal women with breast cancer, not in the premenopausal ones [170-172]. According to the estimation of Secreto *et al*, about 60% of women with breast cancer show some degree of hypertestosteronemia [160;165], and ovariectomy eliminates the T excess, indicating its ovarian origin [160;173-175]. Grattarola found that 40% of breast cancer patients had simple proliferative endometrium, and 43% had hyperplastic or atypical endometrium, virtually pathognomonic for chronic anovulation [176]. It is likely that the hypertestosteronemic and anovulatory populations may be the coincident [160]. Therefore, T excess is likely to be generated from the hyperplastic interestitial cells of the ovary [177], a thought supported by the studies showing that chronic anovulation syndrome is a major risk factor for postmenopausal breast cancer [160;178]. A very high incidence of anovulation in premenopausal breast cancer patients has also been reported [179], although some other studies fail to find a significant association between polycystic ovary syndrome, which is defined by

clinical features of anovulation and hyperandrogenism [180], and the risk of premenopausal breast cancer [181;182]. In some women originally exhibiting T excess, T level is increased again after the ovariectomy; suppression of the adrenal cortex by dexamethasone treatment eliminates the recurrence of T elevation, suggesting that biotransformation of adrenal androgens to T may account for the recurrence [160;173;174;183]. Recent studies not only confirm the strong association of elevated serum concentrations of both E2 and T with increased risk of breast cancer, but further show that the association of free T levels to breast cancer is independent of bioavailable estradiol levels [184;185]. Thus, the mechanism for the association of elevated T with breast cancer seems not as simple as conversion of T to E2.

While the association of T with breast cancer seems to be clear, the relationship between adrenal androgens and breast cancer is still confusing [76;79;170;172;179;186-188]. DHEA, DHEA sulfate (DHEAS), and Adiol (5-androstene-3β,17β-diol) of adrenal origin have all been detected at high amounts in normal breast tissue and in breast cancer [87;115;116;119;179]. Postmenopausal breast cancer patients have been shown to exhibit supranormal plasma levels of DHEA and DHEAS [160;179;187]. Moreover, elevated plasma levels of DHEA have been found in women who subsequently developed postmenopausal breast cancer [150;162;169]. These data imply that similar to T, elevated adrenal androgens may be associated with breast cancer development, as well. Conversely, however, a low urinary excretion of DHEA metabolites has been reported in women who subsequently develop breast cancer, in women with breast cancer, and in women with high risk of the cancer recurrence after mastectomy [160;179;187]. Subnormal plasma levels of DHEA and DHEAS have also been reported in early, as well as advanced breast cancer patients, especially in premenopausal cancer patients [170;171]. These data suggest that higher levels of adrenal androgens may be prophylactic for the development, progression, and reoccurrence of breast cancer.

In cell culture, T and several forms of adrenal androgens have been shown to inhibit growth of many cell lines of breast epithelium or cancer [44;74;134;188-191] but to stimulate growth of several other cell lines [56;192-196]. It has been suggested that androgens stimulate growth of malignant cells more frequently than growth of non-malignant cells, whereas estrogen show an opposite effect [57]. At least for some of the mammary epithelial cell lines, non-aromatizable androgens DHT and 3-α-diol can also stimulate the growth, and estrogen receptor (ER) antagonists cannot block this stimulation. These results suggest that the growth stimulation may not be occurring due to conversion of androgens to estrogens and may not involve ER [56]. Nevertheless, androgens have been shown in many other experiments to exert their effects by binding directly to the estrogen receptor α (ERα) rather than the AR to promote cell proliferation [192;196-199]; in this situation, androgens actually function as estrogens and exert estrogenic effects. Currently, it is still mechanistically unclear how androgens exhibit different roles in the growth of these cells.

Synergy between androgens and estrogens in the tumorigeneity of mammary gland and other organs

In a series of publications since 1950, Kirkman and his co-workers have described that simultaneous administration of estrogens and androgens to Syrian hamsters effectively induces lieomyomas and lieomyosarcomas in the uterus of females and in the epididymal tail and adjacent ductus deferens of males [200-209]. The female animals also exhibit extensive endometrial hyperplasia [201]. The hamsters receiving either estrogen or androgen alone do not develop these tumors, at least at the same time period, although androgen-treated animals develop papillary adenoma of uterus occasionally [202]. Simultaneous administration of androgen and estrogen to

Syrian hamsters of either sex also induces malignant basal cell chaetepithelioma in the flank organs (scent glands) of the skin; this organ consists of many large sebaceous glands, hair follicles, and baskets of melanocytes around the growth cycles of the hair follicles [202;207;209-211]. Androgen is responsible for the early development of the chaetepithelioma but both androgen and estrogen are required for its unlimited growth as a definitive neoplasm.

Noble described in a conference abstract in 1976 that, although Nb rats were relatively insensitive to estrogen-induced mammary carcinogenesis [212], male and female Nb rats receiving subcutaneous implants of both estrone and TP pellets developed mammary carcinomas in approximately 50-60% of the animals [213;214]. Noble also mentioned that estrone treatment of female Nb rats produced mammary carcinomas only when begun in immature rats, whereas combined treatment with both hormones produced carcinomas in rats of any age [212;215;216]. The mammary tumors induced by estrogen or by both estrogen and androgen were transplantable and dependent on estrogen or androgen [217;218]. Although Noble had not published any full report on this rat model of mammary carcinogenesis, he established a prostate carcinogenesis model in male Nb rats by using both TP and estrone pellets [219;220]. According to Noble, administration of TP alone induced prostate cancer at approximately 20% incidence following over one year of treatment [219]. Simultaneous administration of both TP and estrone did not change the incidence of the tumors but shortened their latent period [219;220]. Initial androgen treatment followed by estrogen was the most effective, inducing cancer at about 50% incidence. The prostate tumors induced by both hormones were transplantable [215;216;219;220].

Unaware of Noble's earlier work, Liao *et al* [55] and Xie *et al* [221-224] independently reported similar findings recently, showing that simultaneous administration of TP and E2 (or E2 benzoate in the studies of Xie *et al*) induced invasive mammary adenocarcinomas in male and female Nb rats, at virtually 100% incidence, five to six months following the hormonal treatment. At this time point rats receiving estrogen alone developed only hyperplasia, without tumor formation. Androgen alone does not induce obvious outgrowth of the mammary epithelia at the dose used in these Nb rat models, and therefore is likely to function as a promotional agent for estrogen-induced carcinogenesis. One should not be too surprised by these findings in the Nb rat, since the mammary gland shares the same embryonic origin with the sebaceous gland, the major component of the flank organ in the skin of hamsters, which was shown by Kikman *et al* to develop epitheliomas upon combined treatment of androgen and estrogen [207;210;211]. However, these experimental results in Nb rats raise a concern as to whether a similar synergy between estrogens and androgens also occurs during breast tumorigenesis in humans. Since the literature about whether elevated estrogens are a risk factor of breast cancer has always been inconsistent [4;225-227], it would be an intriguing question as to whether it is the concurrent elevation of both estrogen and androgen, but not each hormone alone, that is a risk factor of breast cancer.

Male-to-female transsexual patients need to receive estrogen therapy to become phenotypic females, and thus exhibit a unique endocrine situation of higher androgen levels than genetic females and higher estrogen levels than genetic males. Cases of the development of mammary cysts, fibroadenomas, and breast cancers have been reported in these transsexuals [228-230]. On the other hand, female-to-male transsexuals need to receive androgens to become phenotypic males while retaining ovaries, which results in higher androgen levels than genetic females and higher estrogen levels than genetic males [131;231;232]. Actually, the female-to-male transsexuals receiving androgen therapy exhibit increases not only in circulating T but also in circulating estrone and E2 [233-235], compared to normal women. This unique endocrine situation may make these female-to-male transsexuals a good population for the study of the possible synergistic tumorigenecity of abnormal estrogen and androgen in women. Unfortunately, so far, there have

only been several studies and case reports on the observations of breast tissue collected at mammoplasty of female-to-male transsexuals [236-239]. In these studies, cystic formation and apocrine metaplasia are the major morphologic findings, while no pronounced hyperplasia nor dysplasia of the mammary epithelia is observed. However, the majority of the cases in these studies received androgen treatment for only one to several years, which is a too-short period relative to the life span in women, considering the fact that hormonal carcinogenesis in experimental animals usually takes one-fourth or more of the life span [55;221]. In addition, many patients in these studies had stopped use of androgens prior to their mammoplasty. Therefore, it remains unknown whether a longer androgen therapy in female-to-male transsexuals associates with neoplastic growth of their mammary glands.

There are also indications for a possible synergistic role of estrogens and androgens in the tumor development of other female reproductive organs. It has been well known that the development of ovarian cysts is associated with elevated levels of circulating T [131;232;240-242]. Since ovarian cysts have a higher risk to progress into ovarian cancer [243-245], elevated androgens may be interrelated to the ovarian cancer development [246-248]. This hypothesis is supported by the facts that AR is expressed in normal ovaries and in most ovarian cancer biopsies [249-252]. Because elevated estrogens have been associated with an increased risk of ovarian cancer [246;247;253], it cannot be excluded that increased estrogen and androgens may actually play a combined role. In line with this consideration, a pronounced increase in the development of polycystic ovarian disease has been documented in the literature for the female-to-male transsexuals, and cases of ovarian cancer have been reported in these transsexuals as well [131;241;254-258].

Elevations in circulating T are associated with the occurrence of endometrial hyperplasia and squamous metaplasia [178;246;259-261]. Since the androgen concentrations in uterine tissue are even higher than the concentrations in the serum [123;262], a direct role of androgens in the uterus is very likely. Supports to this thought are the facts that AR is expressed in significant amounts in endometrium and stroma of normal uterus and in endometrial cancer [134;249;251;263]. Moverover, increased androgenic activity has also been observed in well-differentiated endometrial adenocarcinomas [264]. All these data provide the cellular basis for a direct action of androgens in these normal and malignant uterine tissues.

Cases of endometrial cancer and uterine leiomyomas have been reported in women with hirsutism, usually caused by adrenal disfunction [265-267]. Women with a history of exposure to diethylstilbestrol (DES) in utero, so-called DES daughters, also exhibit an elevated level of ciruculating T [268] and an increase in the frequency of hirsutism [269]. Proliferative endometrium has been observed in the majority of the female-to-male transsexuals receiving androgen therapy, with cystic hyperplasia in some cases [241;256;257]. In line with these clinical data, female mice exposed in utero to DES, a treatment which has been shown to increase circulating T [270], develop uterine adenocarcinomas [84;268;270-276]. Treatment of ovariectomized female rats with T markedly increases the height of the luminal epithelium of the uterus [277] and induces marked endometrial metaplasia and hyperplasia [261]. All of these clinical and experimental results indicate an association of elevated androgens with endometrial outgrowth and cancer. Since elevated estrogens have been associated with the hyperplasia and neoplasia in the uterine endometrium as well [123;246;278-283], it is possible that increased estrogens and androgens may actually play a combined role. Actually, in female Syrian hamsters treated with estrogen and androgen, but not either hormone alone, uterine carcinomas were occasionally discerned, together with cystic glandular hyperplasia of endometrium [208;209]. Xie *et al* also observed a uterine carcinoma in one female Nb rat receiving both hormones, although it was suspected to be a metastasis from the mammary tumors [221].

Possible mechanisms for the roles of androgens in the female reproductive organs

The mechanism accepted by most investigators for the growth-stimulatory action of androgens in mammary epithelia and breast cancer is that androgens serve as precursors for biotransformation to estrogens, resulting in estrogen excess. This mechanism certainly exists and plays certain roles, as described in the above sections. However, it does not explain the growth inhibition by androgens observed in many other situations, and therefore may not be the dominant mechanism, at least in certain circumstances.

It has been proposed for many years that low levels of adrenal androgens may promote breast cancer and higher levels may prevent it [187]. Adrenal androgens are postulated to have two primary effects on mammary tumor cells [76;179;186;188]: 1) In the absence of estrogens, they stimulate growth of breast cancer cells via binding to ERα; this effect can be blocked by treatment with antiestrogen. 2) In the presence of estrogens, they act as antiestrogens to inhibit estrogen-stimulation of growth of breast cancer cells; this effect is exerted via AR and can be blocked by antiandrogens [179;189;284]. According to these hypotheses, in those who have low circulating estrogens, such as most postmenopausal women, androgens may be growth stimulatory for mammary epithelial or cancer cells by direct binding to ERα to mediate estrogenic functions. This line of thinking obtains supports from the *in vitro* observations that androgens, especially those of adrenal origin, indeed are capable of binding to ERα, although the binding affinities are much lower, compared to estrogens [186;192;196-199]. On the other hand, in those who have relatively high circulating estrogens, such as most premenopausal women, androgens may exert mainly antiestrogenic effects via binding to AR, suppressing estrogen-stimulation of the growth of mammary epithelial or cancer cells. Down-regulation of the expression of ERα and progesterone receptor (PR) may be one of the mechanisms for androgens to achieve this effect [76;188;285].

It is a well-known phenomenon that most androgens have various abilities of binding to PR to mediate progestational functions [76;286]. Since progesterone has a complicated influence on the mammary epithelia, including both growth stimulation and inhibition [287], it remains possible that the reported dual functions of androgens may actually be a reflection of their progestational effects. In addition, androgens have been shown to stimulate expression and secretion of prolactin from uterine stromal cells [111] and to induce expression of the prolactin receptor in human breast cancer cells [110]. Neonatal androgenization also increases the secretion of prolactin from pituitary during adulthood [94;106]. Therefore, the effects of androgens may also be a result of increased function of prolactin-prolactin receptor signaling.

The AR gene contains a highly polymorphic CAG trinucleotide repeat, which encodes glutamines, in its first exon. The length of the CAG repeat is inversely associated with the degree of transcriptional activity of AR [288]. In a recently report [289], ovarian cancer patients who carried a short CAG repeat allele of AR was diagnosed on average of 7.2 years earlier than those patients who did not carry a short allele, indicting that a stronger AR activity might be associated with ovarian cancer development. On the other hand, in women who inherit a germline mutation in the BRCA1 gene, those who carry more CAG repeats in at least one AR allele have a higher risk of breast cancer development than those who carry less CAG repeats [290;291]. Since BRCA1 protein is an AR-coactivator that binds to AR and enhances AR signaling [292], it is possible that in BRCA1 carriers, androgens may act via AR to inhibit the development of breast cancer, although it may not be the case in non-BRCA1 carriers.

Another mechanism, which also involves an AR-coactivator but not androgens *per se*, is that in the presence of the AR-coactivator ARA_{70}, E_2 (but not DES) is capable of binding to AR

and activating AR transcriptional activities in some human prostate cancer cells [293;294]. Evidence has also been shown that this mechanism may be involved in the development of the male reproductive system as well [293]. Thus, the natural estrogen can also function as an androgen to be a natural ligand of AR under certain, yet undefined, conditions. It is currently unknown whether such ARA_{70}-mediated activation of AR transcriptional activities by E_2 also occurs in the normal and malignant tissues of the mammary gland and other female reproductive organs, although ARA_{70} expression has recently been shown to be activated in human ovarian cancer [295].

In summary, androgens of both ovarian- and adrenal-origins have been shown, both in animals and in cultured cells, to either stimulate or inhibit growth of the mammary gland and breast cancer. There are at least six possible mechanisms for androgens to accomplish these functions: 1) Androgens serve as estrogen precursors and are converted to estrogens. 2) Androgens exert estrogenic effects by directly binding to ERα; adrenal androgens have higher affinities for ERα than T and DHT, and therefore are more potent in this function. 3) Androgens exert androgenic effects by directly binding to AR. 4) Androgens may bind to PR and exert progestational effects. 5) Androgens may stimulate the expression of prolactin and prolactin receptors, playing the functions of prolactin. 6) In the cases of BRCA1 carriers, androgens may act via AR-BRCA1 complex to inhibit the development of breast cancer; this mechanism, if it really exists, is affected by the length of the CAG repeat in the AR gene.

Perspectives

Many references described in this review seem to lead us to a hypothesis that increased androgens and estrogens may be synergistic in tumorigenecity of several tissues of the reproductive systems. So far, this hypothesis has received experimental evidence in rodents for tumorigenesis in the prostate, epidimis, uterine myometrium, and mammary gland, as described above, while direct experimental evidence for tumorigenesis in the ovary and endometrium is still lacking. Attempts have been made to use either estrogen or androgen alone, but not in combination, in the induction of ovarian cancer and endometrial cancer [207;274;296-301]. The results of uterine carcinogenesis by either estrogen [274;275;297;300] or androgen [296;301] have been satisfactory, at least in certain strains of mice. Combined treatment of DMBA and T, but not each alone, has also been shown to effectively induce endometrial cancer in female rats [302]. However, estrogen- or androgen-induced ovarian tumorigenesis has thus far resulted mainly in benign epithelial neoplasms, at least in guinea pigs [298;299]. It would be intriguing to see whether combinations of androgen and estrogen at certain doses and ratios are capable of inducing tumors of the ovary and endometrium at high incidences, and which forms of androgens and estrogens are more potent than the others for tumorigenecity. It is possible that even if androgens and estrogens are eventually shown to be indeed synergistic for tumorigenecity of many reproductive organs, including the ovary and endometrium, the optimal doses of each hormone and the ratios between the two hormones may be tissue-specific. Different forms of androgens and estrogens may show different tumorigenic potencies for different tissues or organs. Each specific target tissue may have its own optimal form of androgen and estrogen and its most optimal ratio of the two hormones for tumorigenecity. This may explain the observation that uterine carcinomas develop only occasionally in the female Syrian hamsters [208;209] and Nb rats [221] treated for other purposes with both estrogen and androgen.

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